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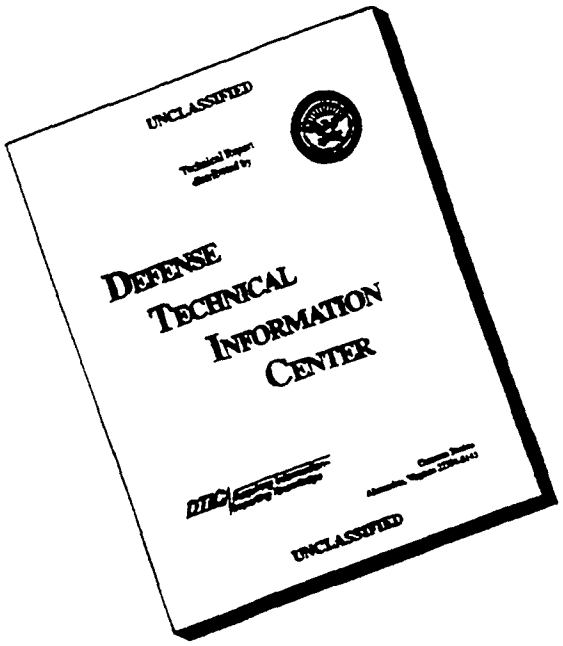

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Significant progress has been made during year 02. DNA vaccine experiments continue in the cost-effective mouse model involving Rauscher murine leukemia virus (RLV), in parallel to experiments in the SIV/rhesus monkey system. New RLV DNA vectors have been constructed, and expression of viral gene products has been documented by transient and stable transfections. CTL assays have been established, and a pilot study involving various DNA vaccines in mice has been conducted.

A few months ago, we entered into an agreement with the Yerkes Regional Primate Research Center, which maintains a large rhesus monkey breeding colony. This has given us excellent access to neonatal animals. In collaboration with Dr. Harriet Robinson, a large DNA vaccine study has been initiated in neonatal macaques, which parallels an experiment that is ongoing in adults, which gives us an opportunity to test whether a given DNA vaccine exhibits age differences in immunogencity. Our key finding is that many macaque infants have developed specific antibodies in response to DNA vaccines. This indicates that the neonatal immune system, rather than being tolerized, is capable of responding to DNA vaccines. We continue to characterize the immune responses further. Challenge experiments with live virus are planned.

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FOREWORD

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INTRODUCTION

The goal of this project is to develop DNA vaccines to treat and/or prevent primate lentivirus infection. The individual specific aims have been adjusted during year 02 of this project. We have had the excellent opportunity to enter into an agreement with the Yerkes Regional Primate Research Center (Atlanta, Georgia), which maintains a large rhesus monkey breeding colony. This group of animals has proven to be free of adventitious retroviral infection; highly sensitive polymerase chain reaction (PCR) assays as well as serological surveys have found no evidence of exposure of these animals to either simian retroviruses type D (SRV/D), simian T-lymphotropic virus type-I (STLV-I) or simian immunodeficiency virus (SIV).

As stated in the Annual Report of 1995, our previous subcontractor, TSI Mason Laboratories, has not been able to provide us with the requisite number of pregnant rhesus macaque dams during year 01 of this project. Consequently, the monies designated for the primate subcontractor of years 01 and 02 were combined. This allowed us to initiate a large DNA vaccine study, in collaboration with Dr. Harriet Robinson (University of Massachusetts, Worcester). A total of 28 newborn rhesus monkeys were given various DNA vaccines or control vector. The most exciting aspect of this vaccine trial is the fact that a parallel study has been initiated by Dr. Robinson in adult/juvenile rhesus macaques in a separate vaccine trial, using some of the same vaccine strategies that are being evaluated in our neonatal rhesus macaque experiment. <u>This experimental plan gives us the unique opportunity to test whether DNA vaccines exhibit age-related differences in immunogenicity.</u> If the vaccines are found to be safe as well as immunogenic in neonatal primates, important information will have been generated for applying this novel technology world-wide for childhood vaccination.

Our vaccine study in neonatal macaques is also asking the question whether protective immune responses can be elicited in the absence of antibodies directed against the glycoprotein envelopes. Some test animals have only been given DNA vaccines encoding interval viral gene products, but not envelope glycoproteins.

The dose, frequency, and route of DNA administration closely parallels the experimental design used for vaccinating the adult/juvenile macaques. A total of 4 DNA inoculations will be given over a period of 1 year. As this is a relatively prolonged vaccination schedule, we tested in our mouse model which involves the Rauscher murine leukemia virus (RLV) whether a short vaccination protocol could produce protective immunity.

Groups of mice were vaccinated by the intradermal or intramuscular route with DNA expression vectors encoding various RLV gene product. Some animals were given one boost, whereas others were not boosted. All animals were rechallenged with 20 animal infectious doses of live RLV. No protection was seen with this abbreviated vaccine schedule. In fact, there may be a tendency for vaccine-induced enhancement of infection in some but not other vaccine strategies (see below).

For the third year, we plan to continue to evaluate and characterize the immune responses generated in the infant rhesus macaques. These animals continue to be followed in parallel to the vaccines involved in the adult/juvenile macaque study. The animals will be challenged approximately a year after receiving the first vaccine dose, which will be during the Spring/Summer months of 1997.

Progress has been made also during year 02 on another aspect that will be a major benefit to primate vaccine research. We have developed highly sensitive and specific PCR tests to identify animals exposed to SRV/D or STLV-I, but showing no evidence of seroconversion. The availability of these tests will allow primate centers to eliminate endemic infection with these viral agents.

We plan to continue to exploit the low-cost RLV system in attempts to find vaccine strategies that will protect the animals against infection when challenged with live virus. A more detailed description of the individual experiments is given below.

Publications in Year 02:

1. Baba TW, Liska V, Hu Y, Rasmussen RA, Penninck D, Bronson R, Greene MF, Ruprecht RM. Attenuated retrovirus vaccines and AIDS. Science 1995; 270:1220-1222.
2. Ruprecht RM, Baba TW, Li A, Ayehunie S, Hu Y, Liska V, Rasmussen R, Sharma PL. Live attenuated HIV as a vaccine for AIDS: Pros and cons. Seminars in Virology 1996; 7:147-155.
3. Ruprecht R, Hu Y, Liska V, Rasmussen R, Sharma P. The correlates of immune protection after attenuated live MuLV vaccination. AIDS Research and Human Retroviruses, 1996; 12:375-377.
4. Ruprecht RM, Baba TW. Murine and simian retrovirus models: The threshold hypothesis. AIDS 1996; 10 (suppl A):S33-S40.
5. Ruprecht RM, Baba TW, Ayehunie S, Liska V, Montefiori DC, Rasmussen R. SIV pathogenesis during ontogeny: the influence of host factors. In: O. de Garidel, V-A Libert, eds., 10e COLLOQUE DES "CENT GARDES" Retroviruses of Human A.I.D.S. and Related Animal Diseases. 23-24-25 October 1995:229-234.
6. Liska V, Lerche NW, Ruprecht RM. Simultaneous detection of simian retrovirus type D serotypes 1, 2, and 3 by polymerase chain reaction. AIDS Research and Human Retroviruses, in press.
7. Ruprecht RM, Rasmussen RA, Hu Y. Induction of protective immunity by exposure to low doses of a live, pathogenic retrovirus. Manuscript in preparation.
8. Liska V, Fultz PN, Su L, Ruprecht RM. Prolonged STLV-I infection without seroconversion. Manuscript in preparation.

MATERIALS AND METHODS

1. Sequencing the RLV genome. The complete RLV genome construct, p3028N, which encodes the replication-competent helper virus, was used as template for sequencing. Primers were designed from published Friend murine leukemia virus (F-MuLV), strain FB29, sequence data (1), since a high degree of homology was expected between the two genomes. The sequence data were obtained on the double-stranded plasmid DNA construct p3028N using dye terminator on the Applied Biosystems automated sequencer model 373A. Both strands were sequenced (see also in the Appendix).

2. Construction of RLV expression plasmids. Based on the complete R-MuLV sequence above primers were made flanking the *gag*, *pol* and *env* regions of the genome with convenient restriction sites at each end to facilitate cloning into the expression vector, pJW4303 (Fig. 2A & B). pJW4303 was developed by Dr. Jim Arthos (NIH, Bethesda, MD) and kindly provided to us by Dr. Jim Mullins (University of Washington School of Medicine, Seattle, WA). This vector has a strong CMV immediate early promoter and bovine growth hormone polyadenylation signal (2). The following regions of the R-MuLV genome were successfully amplified by long PCR for cloning in the vector (Fig. 3): *gag, gag-protease* (*gag-prot*), *gag-pol* and *env.* The amplified *gag-prot* and *env* genes were successfully inserted in the cloning site Hind III and Nhe I (downstream of the CMV promoter) of the vector, pJW4303 (Fig. 2B). Recombinant pJW*gag-prot* and pJW*env* DNA from selected clones were grown in large amounts using the Qiagen Megaprep Kit (Qiagen, CA).

3. Transient transfection of RLV expression plasmids. Before vaccination of mice, the DNA expression vectors were tested in vitro by immunoprecipitation of Cos cell transfectants. Cos cells were transfected with each plasmid (1 mg DNA/ml) using DEAE-dextran and 72 hrs later, the cells were harvested, and membrane lysates were prepared in a 1% NP-40 lysate buffer containing protease inhibitors. Nuclei and debris were pelleted and lysates were then incubated with Protein A-Sepharose beads that had been preincubated with either anti-Env or anti-Gag monoclonal antibodies (mAbs). After washing, specific antigens were eluted from the beads by boiling in SDS-PAGE sample buffer and were separated by gel electrophoresis. Proteins were transfered to nitrocellulose membranes and the immunoprecipitated antigens were detected by incubating the membrane with goat anti-RLV antiserum followed by horseradish peroxidase-conjugated rabbit anti goat IgG.

4. Stable transfection amd generation of RLV-env-expressing murine cells. We have also used the pJW*env* plasmid to establish permanent transfectants of P815 cells. Cells were cotransfected with the RSV-2neo plasmid, and G418-resistant cells were selected in drug-containing medium. Transfected P815 cells were stained using an anti-Env IgG.

5. Mice and virus. Six-to eight-week-old female BALB/c mice (Taconic Farms, Germantown, NY) were used for all experiments. Rauscher murine leukemia virus

(RLV), strain RV-B, derived from the original stock, had been prepared by tail vein injection of 10^4 plaque-forming units (PFU) of RLV into mice. Single cell suspensions of spleens obtained from animals sacrificed on day 20 post-inoculation had been prepared in medium (2 ml/g spleen) supplemented with 20% fetal calf serum (FCS), and cell supernatants had been stored in liquid N_2. The number of PFU in the stock had been determined by XC plaque assay, and the stock had been titrated also in mice (3).

6. Immunoblot analysis for RLV. Total protein of serum samples was measured using Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA). Twenty μg of serum protein were separated by 10% SDS-PAGE and transblotted onto Immobilon-P membranes (Millipore, Bedford, MA). After blocking nonspecific binding sites with 0.1% Tween, the membranes were incubated first with goat anti-RLV antiserum (NIH Repository Number 75S000294) and, after washing, with horseradish peroxidase-conjugated rabbit anti-goat IgG (Bio-Rad, Hercules, CA). Specific binding was demonstrated after the blot was developed with the substrate 4-chloro-1-napthol (Life Technologies, Inc., Gaithersburg, MD). Individual lanes were scored positive for RLV by the presence of p30 Gag, p15E and gp70 Env bands.

7. CTL responses in RLV-immune mice. A specific CTL assay has been developed to measure cytotoxicity of immunized BALB/c mice against RLV-infected target cells. Such cells were prepared by incubating P815 cells (BALB/c mastocytoma cell line) with RLV (5×10^4 PFU/10^6 cells) in the presence of Polybrene (Sigma Chemical; 8 mg/ml) overnight at 37°C in culture media (Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 10 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin). The cells were expanded and stained for RLV Env and Gag antigen expression using anti-gp70 Env mAb 48, and anti-p30 Gag mAb R187 (both from ATCC), which were developed against Friend MuLV but cross-react with RLV antigens. RLV-infected P815 cells were sorted by flow cytometry for Env expression, expanded and routinely checked for Env and Gag antigen expression, which was maintained at >90%. The RLV-infected P815 cells were termed P815-R.

To measure specific cytotoxicity, RLV-infected or noninfected P815 cells were radiolabelled by incubation with $Na^{51}CrO_4$ (new England Nuclear; 100 μCi/10^6 cells) for 1 hr at 37°C, then washed and incubated with the indicated effector cells (obtained from mice inoculated with live, drug-"attenuated" virus) in 96-well round-bottom plates. After 5 h, supernatants were harvested onto SCS harvesting frames (Skatron Inc., Sterling, VA) and released ^{51}Cr measured in a gamma-counter. Assays were performed in triplicate, and the percent specific cytotoxicity was determined according to the formula:

$$\frac{\text{(experimental release - spontaneous release)}}{\text{(total release - spontaneous release)}} \times 100$$

where spontaneous release is that from target cells cultured in medium alone and total release that from target cells cultured in 1% NP-40 detergent.

8. Vaccination of mice with anti-RLV DNA vaccines. After a one-week quarantine, the mice were started on the vaccine protocol as outlined on Table II. On day 0, the indicated number of mice per group received the DNAs indicated either intradermally (id) or intramuscularly (im). Some groups of mice were treated with intraperitoneal (ip) injections of interleukin (IL)-12 between days 0-4, at 1 μg/mouse/day for a total of 5 injections. On day 14, some groups of mice were given booster injections of DNA. On day 20, all mice were challenged by intravenous (iv) injection of RLV at 20 50% animal infectious doses (AID_{50}). On day 41, serum samples were collected for immunoblot assay to test for the presence of RLV antigens. On day 51, all mice were sacrificed, and their spleens were weighed.

9. Generation of an ELISA assay to measure anti-RLV antibodies. An assay to measure humoral responses in RLV-immunized mice using a cell ELISA employing P815-R cells (and their uninfected counterparts as controls) has been established.

10. DNA vaccination of neonatal rhesus monkeys. Groups of 4 rhesus macaque neonates were enrolled into a vaccine protocol that involves 7 groups. Prior to delivery, the pregnant dams were pre-screened by serology and PCR to rule out subclinical infection with simian retroviruses (SIV, SRV/D and STLV-I). The offspring were delivered by natural vaginal birth, and the dams were returned to their breeding colonies. The neonatal macaques were given the DNA vaccines as outlined (Table II) within the first few days of life. Animals enrolled in groups A through E were entered into trial according to a computer- generated randomization schema. Because the births occurred over a period of several weeks, this strategy was adopted to avoid potential bias because of season or other factors. Because the staff at the Yerkes Regional Primate Research Center needed to be trained specifically in the correct use of the gene gun by a member of Dr. Harriet Robinson's team, the animals in groups F and G were all vaccinated within a time span of eight days.

The neonatal animals were hand-reared. Booster injections of DNA plasmids were administered at 4 weeks, and will be given at 6 and 11 months. Blood samples were collected at birth, at 2 weeks, 6 weeks, and at 5 1/2 months. Further blood collections will be taken at 6 1/2/ and 10 1/2 months. Antibody levels are being followed by ELISA, Western blot analysis and neutralization of autologous virus. CTL activity will be measured at 5 1/2, 6 1/2 and 10 1/2 months. If the DNA vaccines are found to be immunogenic in the infant macaques, the animals will be challenged with live virus at approximately one year.

11. Western blot analysis of specific anti-SHIV immune responses. Commercially available HIV-1 Western blot strips were used to measure anti-HIV-1 Env responses. Because the Gag antigens are well conserved between HIV-1 and SIV, we expect to find anti-Gag responses by this Western blot analysis as well; however, the serum

samples will be tested for anti-SIV responses also by commercially available HIV-2 strips, which we have used extensively for our previous SIV work.

12. SIV-specific CTL responses in macaques. CTL assays in the rhesus macaque system were established by evaluating adult macaques infected with SIVmac251.

12a. Recombinant virus vectors. Recombinant vaccinia viruses containing wild type vaccinia or *gag*, *pol*, or *nef* genes of SIV have been obtained from Dr. Panicali (Therion Biologics Corp., Cambridge, MA). The appropriate recombinant vaccinia viruses to measure CTL activity in SHIV-DNA-vaccinated animals have been obtained also.

12b. Preparation and labelling of B-lymphoblastoid cell lines (B-LCL). Peripheral blood samples were collected in sterile, preservative-free heparin and shipped to the Dana-Farber Cancer Institute on ice. Peripheral blood mononuclear cells (PBMC) were separated on Ficoll-hypaque gradients and infected with cell-free herpesvirus *papio*. Autologous B-LCL target cells were labeled with ^{51}chromium and infected overnight with wild-type vaccinia or recombinant vaccinia vectors containing either a single *gag/pol/env* gene insert, or individual *gag*, *nef*, or *env* SIV gene inserts.

12c. CTL effector cell cultures. PBMC from monkeys testing negative for all non-simian retroviruses were obtained by Ficoll-hypaque gradient centrifugation of blood. PBMC were cultured in RPMI-1640 media supplemented with 15% FCS, penicillin, streptomycin, and L-glutamine. Mitogen-stimulated PBMC effector cells were cultured in medium containing a 5 μg/ml Concanavalin A (ConA) for 3 days, washed and cultured for four days in medium containing 10% human IL-2. Other cultures were stimulated for two days with autologous paraformaldehyde-fixed B-LCL infected with a vaccinia vector containing an SIV *gag/pol/env* gene construct, and then cultured for five days in medium containing 10% human IL-2.

12d. SIV-specific cytotoxic CTL assays. SIV-specific CTL activity was determined using various effector-to-target cell (E:T) ratios in a standard 5 h ^{51}Cr release assay. The percentage of specific cytotoxicity was determined from the formula: 100 (experimental release - spontaneous release)/(maximum release - spontaneous release).

12e. Immunomagnetic cell separation. Effector PBMC were isolated on Ficoll-Hypaque gradients and separated by immunomagnetic selection using Dynal beads coated with either anti-CD4 or anti-CD8 antibodies using instructions provided by the manufacturer. Cells were released, washed, and used in CTL assays as described above. An aliquot of these cells was used to assess the purity of the selected cell subsets by flow cytometry.

RESULTS

For easy review, the progress made during the past year is broken down according to the scientific topics. First, we will briefly summarize work conducted in the RLV/mouse system. Subsequently, the large DNA vaccine trial in infant rhesus macaques will be summarized. The discussion will also include a brief summary of the PCR assays that have been generated to rule out clinically silent infection with SRV/D and STLV-I. Lastly, the plans for the coming year will be highlighted.

1. Determination of the DNA sequence of the entire RLV genome.

During year 02, we have completed the sequence analysis of the RLV genome; both proviral strands have been sequenced. The gene bank accession number for submitting the complete sequence will be obtained within the next few days. The entire sequence of the replication-competent Rauscher murine leukemia virus helper virus, termed R-MuLV, is 8,282 bases in length. The overall nucleotide sequence identity between R-MuLV and the Friend murine leukemia virus (F-MuLV) was found to be 97.6% (Table I) as determined the GAP alignment program in CCG (Genetics Computer Group, Madison, WI). This high degree of homology between R-MuLV and F-MuLV was expected as the two viruses exhibit a similar pathobiology. Based on the alignment of the Gag, Pol and Env proteins of R-MuLV and other murine leukemia viruses, phylogenetic trees were constructed to determine the evolutionary relationship between individual genes of these viruses (Figure 1). The three trees were generated by using the PIMA (Pattern-Induced Multi-Sequence Alignment) algorithm (4) and PAUP software (5) based on position 3 of the codons in the *gag*, *pol* and *env* regions of all the viruses listed in the comparison. The Gag tree shows that R-MuLV and F-MuLV evolved more slowly, possibly due to some selective constraint. However, the two viruses fall into one very closely related subgroup when compared to the remainder of the tree.

The R-MuLV sequence data and its phylogenetic analysis with sequences of other murine leukemia viruses will be submitted in a manuscript entitled: "The complete genome of Rauscher murine leukemia virus and its phylogenetic relationship with other murine leukemia viruses". A copy of this manuscript has been included in the Appendix.

2. Construction of RLV expression vectors.

The complete R-MuLV sequence proved to be helpful in the construction of expression vectors encoding the *gag, pol* and *env* regions. Primers were synthesized flanking these coding regions with convenient restriction sites at each end to facilitate cloning into the expression vector, pJW4303 (Figures 2A and 2B). pJW4303 had been developed by Dr. J. Arthos (NIH, Bethesda, MD) and was kindly provided to us by Dr. Jim Mullins (University of Washington School of Medicine, Seattle, WA). This vector has a strong cytomegalovirus (CMV), immediate early promoter and bovine growth hormone polyadenylation signal (2). Long-distance PCR was successful in amplifying the *gag, gag-protease (gag-prot), gag-pol* and *env* regions into the expression vector, using the *Hind* III and *Nhe* I sites downstream of the CMV promotor in the pJW4303

vector (Figures 2B and 3). Milligram quantities of pJW*gag-prot* and pJW*env* plasmid DNA were generated from single clones using the Qiagen Megaprep kit (Qiagen, CA) for testing as DNA vaccines.

3. Transient and stable expression of RLV genes from the expression vectors.
The RLV DNA expression vectors were tested for their ability to express the viral gene products by transient transfection by Cos cells, followed by immunoprecipitation. The results of one such immunoprecipitation are shown in Figure 4. These demonstrate clearly that the Env and Gag proteins are expressed in mammalian cells after transient transfection with pJW*env* or pJW*gag-prot,* respectively. We have also used the pJW*env* plasmid to establish permanent transfectants of murine P815 cells. To generate these cell lines, P815 cells were cotransfected with the RSV-2neo plasmid, and neomycin-resistant cells were selected in G-418-containing medium. The selected cells were stained using an anti-Env IgG; by flow cytometry, a low level surface expression of RLV-Env was detected (Figure 5).

4. Establishment of anti-RLV CTL assay.
To generate syngeneic target cells, P815 cells (which had been derived from BALB/c mice) were infected with RLV, giving rise to P815-R cells. As this virus is not cytolytic, an infection of 100% could be obtained in vitro, and all cells were infectious by the XC infectious center assay and expressed high levels of Gag and Env protein on their cell surfaces. To test whether these cells could serve as targets for CTL assays, we generated RLV-immune mice according to our well established protocols involving live attenuated virus (3). Young adult mice were inoculated intravenously with high-dose RLV and treated with antiviral chemoprophylaxis (using AZT and interferon-α) for 20 days. We had shown previously that this regimen prevents viremia and disease in all mice (3). Furthermore, the virus-inoculated, drug-treated mice developed protective immunity when we challenged with high doses of live virus (3). At various time points after inoculation during the drug treatment, mice were sacrificed, and their spleen cells were tested for RLV-specific CTL activity in standard 5 h chromium release assays. As shown in Figure 6, CTL activity was readily detected starting on day 11 post-inoculation and peaked on day 21. The CTL response to RLV could be shown to be mediated predominantly by $CD8^+$ T-cells (Figure 7).

5. Establishment of an ELISA to measure anti-RLV antibody levels.
To measure humoral immune responses in RLV-vaccinated mice, we have established an ELISA employing P815-R cells. As shown in Figure 8, mouse serum from mice inoculated with RLV and treated with post-exposure chemoprophylaxis exhibited strong antibody reactions against RLV-infected cells.

6. DNA vaccine experiments.
During year 02, two separate DNA vaccine trials have been conducted, one in mice in the RLV system, and the second one in neonatal rhesus macaques. The latter experiment was made possible by our new affiliation with the Yerkes Regional Primate Research Center, which provided us access to a large number of retrovirus-free

pregnant macaques. We had the additional opportunity to conduct a DNA vaccine study in neonatal macaques that parallels a study that had been started already in juvenile/adult macaques by our collaborator Dr. Harriet Robinson (University of Massachusetts, Worcester, MA). This allowed us to ask the following questions: Can neonatal primates respond immunologically to DNA vaccines, or will they be tolerized? Are there qualitative and quantitative changes in the immunogenicity of DNA vaccines in neonatal and adult primates? Ultimately, can DNA vaccines generate protective immune responses in neonatal and adult rhesus macaques? A brief summary of the two vaccine experiments is given below.

6a. Short-term anti-RLV vaccination strategy in mice.

Because the primate DNA vaccine experiment involves a long-term vaccine strategy, we decided to test in the mouse system whether a short-term vaccination schedule could induce protection against virus challenge. Similar approaches had been found to protect mice against challenge with rabies virus (Nancy Ray, unpublished observation). We also asked whether the use of systemic IL-12 would provide better protection through the induction of cellular immunity against RLV. The data are summarized in Table II.

Surprisingly, we found that not only did the DNA vaccines fail to provide protection against infection, as evidenced by the universally positive immunoblots after virus challenge with 20 AID_{50}, but they also led to enhancement of infection in the groups that were vaccinated with the pJW*env* vector intradermally, as measured by the degree of splenomegaly when compared to mice infected with the vector only (Group D versus group B, Table II). In the RLV system, the degree of splenomegaly seen 2-3 weeks post-inoculation is proportional to the level of virus. In group D, a statistically significant enhancement was seen, even after a Bonferroni correction for multiple comparisons (p significant at $\leq$ 0.017) was made. The spleen weights in groups C and E (in which the combination of 1 μg of each pJW*env* and pJW*gag-prot* were tested) are suggestive of an enhancing effect, but statistical significance has not been reached.

Groups F through I were enrolled into the pilot study to assess vaccine dose effects. Given the wide fluctuations in spleen weights, we can not detect any dose-response relationship. No statistically significant differences were observed between groups I and J, in which we tested the effect of IL-12 given for 5 consecutive days after DNA vaccination.

Mice in groups K and L were enrolled into a pilot study to assess the effect of boosting with the combination of DNA expression vectors. While no protection was seen, the infection-enhancing effect observed in group B was not present either. Possibly, intradermal vaccination with *env*-expressing DNA vectors at 1 μg per mouse induces infection-enhancing immune responses, whereas boosting may induce protective responses. We postulate that in group E, to which only one single dose of DNA vaccine was given, no protective immune responses were present, whereas in

group L, the infection-enhancing and protective immune responses canceled each other.

Lastly, in groups M, N, and O, we tested high-dose DNA vaccinations (100 μg per animal) through the intramuscular route, using again the combination of pJW*env* and pJW*gag-prot*. Group O differed from group N by the addition of IL-12 treatment for five days. Group N again showed enhancement of the infection as assessed by a significantly greater degree of splenomegaly as compared to group M. In contrast, this enhancement was abrogated by the addition of IL-12 to the DNA vaccine.

At first glance, the vaccine effect of the new generation of DNA expression vectors appears disappointing in this pilot study. However, a few important trends can be dissected from the information of the pilot experiment. First, intradermal vaccination combined with boosting showed no infection enhancement, in contrast to the trend observed in the absence of boosting. Second, intramuscular vaccination with high doses of DNA also resulted in enhancement, which was not seen when IL-12 was co-administered with the vaccine. It should be stressed that this pilot study was designed to examine whether short-term vaccine strategies could yield protection. As this is obviously not the case, our subsequent studies will examine strategies involving multiple boosts, spread over long time periods, as we are testing currently in the primate model. Co-administration of IL-12 will be examined as well.

In sum, further studies will test our hypothesis that repeated boosting will lead to a maturation of immune responses; we postulate that infection-enhancing responses will be replaced by protective immunity induced by repeated boosting and/or co-administration of IL-12.

6b. DNA vaccine studies in neonatal rhesus macaques.

As stated in previous sections, the disappointment we suffered in year 01, during which we were unable to obtain the requisite numbers of pregnant macaque dams, prompted us to switch our affiliation from TSI Mason Laboratories to the Yerkes Regional Primate Research Center. This new affiliation has given us the unique opportunity to conduct a relatively large vaccine study in the neonatal macaques, as the monies from year 01 were reallocated to year 02 and as Yerkes was able to provide us with large numbers of retrovirus-free pregnant macaque dams. Another major plus is our collaboration with Dr. Harriet Robinson (University of Massachusetts, Worcester, MA), who has initiated a DNA vaccine study in juvenile/adult macaques using DNA expression vectors encoding various gene products of the chimeric SHIV virus. This virus, which was built from the genome of SIVmac$_{239}$, contains the *vpu*, *tat*, *rev*, and *env* genes of HIV-IIIB. Dr. Robinson and myself decided to design a DNA vaccine study in neonatal macaques using the same DNA expression vectors at the same doses and routes of administration that were used in her ongoing study. Consequently, groups 1, 5 and 6 represent exact replicates of her ongoing study, albeit in neonates. In contrast, groups 2, 3, 4, and 7 are unique to our study in neonates.

The first novel question that we sought to address experimentally is concerning the need for neutralizing antibodies directed against envelope glycoproteins in generating protective immune responses against live-virus challenge. To address this, animals in group 3 were vaccinated only with expression vectors encoding *gag-pol* and *nef*. The DNA vaccines will be administered intradermally according to the same schedule used for groups 1 and 5. The animals will be followed prospectively for the development of humoral and cellular immune responses as all other experimental animals. We do not expect that neutralizing antibodies will be generated, given the absence of *env*-encoding DNA plasmids.

The second issue that is being addressed solely in our neonatal vaccine experiment concerns the role of IL-12 given systemically for short periods of time with DNA vaccines. Can short pulses of IL-12 therapy skew immune responses towards cellular immunity? Will we discern differences in the rate of protection, once the animals are challenged during the following year? To test this, animals in groups 2, 4, and 7 have been vaccinated as outlined in Table III but are undergoing short courses of IL-12 therapy (given intradermally with each administration of DNA vaccine for five days). With approximately 5 1/2 months of follow-up, we cannot yet make any statements regarding differences in immunogenicity. Because very little blood can be obtained from neonatal rhesus monkeys, CTL assays will only be conducted at approximately six months of follow-up. Consequently, no CTL data have been generated yet.

Examination of humoral immune responses in the vaccinees by Western blot analysis has revealed that nine out of 24 animals have seroconverted, as evidenced by the appearance of specific bands on HIV-1 Western blot strips. Analysis using a commercially available HIV-2 strips is pending.

7. CTL studies in DNA vaccinated infant rhesus macaques.

In addition to the 28 infant macaques enrolled in the DNA vaccine trial (one animal has died due to neonatal complications and was replaced), two additional mother-infant pairs have been infected with $SIVmac_{251}$ in order to provide a system for evaluating CTL assays. We have been able to establish CTL assays in our own laboratory (Figure 10). Both adult macaques infected with $SIVmac_{251}$ exhibited specific lysis in a standard chromium release assay. The background cytotoxicity with wild-type vaccinia virus was less than 10% for both animals, whereas the specific killing was approximately 15%. We have also developed the capability of freezing and thawing effector cells, which is important in managing an experimental group of 28 vaccinees, which represents a very high work load.

We have established B-LCL on all rhesus macaque infants enrolled into the vaccine trial. Multiple aliquots of these cells have been frozen down. The relevant vaccinia virus constructs expressing SIV and/or HIV-IIIB antigens have been obtained through the help of Dr. Fred Vogel (NIAID). We are now poised to start a systematic analysis of CTL responses in our cohort of vaccinees.

8. Establishment of a diagnostic, highly sensitivity PCR assay to rule out subclinical infection with SRV/D.

We have succeeded in establishing a very sensitive and specific assay that allows the simultaneous detection of SIV serotypes 1, 2, and 3 by DNA PCR. The assay system allows detection of approximately one proviral copy in 150,000 cells (Liska, et al., AIDS Research and Human Retroviruses, in press, please see also Appendix). We are now using this test routinely to prescreen all experimental animals. We have also made the test available to any interested primate researchers, including investigators at European primate centers. This fast, easy and sensitive assay will be of great help in colony management.

9. PCR assay to rule out seronegative infection with STLV-I.

A highly sensitive and specific PCR diagnostic test has been developed to document low levels of STLV-I infection in primates. In collaboration with Dr. Patricia Fultz, we have found that STLV-I, like other retroviruses, can remain serologically silent for prolonged periods of time. Consequently, antibody assays are not sufficient to rule out infection. This new test will be published shortly and will be made available to any interested primate research center.

SUMMARY AND PLANS

Significant progress has been made during year 02, and important new data have been generated both in the mouse as well as in the macaque model systems. Compared to live attenuated (and even low-dose live, live pathogenic) virus vaccines, the new generation of pJW RLV expression vectors administered as DNA vaccines in a short-course vaccine strategy in mice were a disappointment. We plan to broaden the antiviral immune response by generating defective virus particles. These can be generated from an expression vector encoding the entire RLV genome containing a *pol* deletion. The short-term vaccine strategy will be replaced by a schedule involving multiple boosts, given over prolonged periods of time. Antibody and CTL responses will be measured prospectively.

The primate DNA vaccine study has revealed the exciting finding that DNA vaccines are immunogenic, even when administered within the first few days of life. The large-scale study in progress has been designed to address some important issues, such as: 1) Are there age-related differences in DNA vaccine immunogenicity and protective responses? 2) Can protective responses be generated in the absence of anti-envelope glycoprotein envelope antibodies? 3) Does the co-administration of IL-12 favor the generation of cellular immune responses? Are IL-12 treated vaccinees better protected against virus rechallenge?

During year 03, we plan to continue our vaccine schedule in the primates. If we find significant immune responses, as measured both by antibody and CTL responses, the animals will be challenged during the next year.

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Table I

	Strain	U3	R	U5	5' Leader[g]	*gag*		*pol*		*env*		Complete Genome
						nt[h]	a.a.[i]	nt	a.a.	nt	a.a.	
F-MuLV	PVC-211[a]	97.3	98.5	100	96.4	98.0	97.4	97.9	98.5	96.9	95.4	97.6
	57[b]	97.3	93.0	100	96.8	97.7	96.7	97.5	98.0	96.9	96.3	97.3
	FB29[c]	97.6	98.5	98.7	95.9	97.6	96.5	98.1	98.3	96.9	95.7	97.6
Mo-MuLV[d]		86.33	94.1	94.8	91.6	84.8	90.7	90.9	96.1	82.0	85.7	87.6
Cas-Br-E-[e]		88.9	97.0	97.3	90.3	84.8	92.5	91.6	96.5	72.0	77.9	85.7
Akv[f]		74.8	95.6	90.7	74.2	79.2	85.3	84.7	89.8	75.7	80.5	80.8

Legend Table I. Sequence homology between the R-MuLV and other murine leukemia virus genomes.

[a]Accession #M93134 (4).
[b]Accession #X02794 (5).
[c]Accession #Z11128 (6).
[d]Accession #J02255 (7).
[e]Accession #X57540 (8).
[f]Accession #J01998 (9, 10).
[g]5′ leader sequence from the 3′ end of U5 to the start codon of *gag*.
[h]nt: percent nucleotide identity.
[i]a.a. percent amino acid identity.

Table II

PILOT STUDY OF DNA VACCINES AGAINST RLV

Group #	# of mice	DNA		IL-12	RLV	Serum IB (positive)	Mean spleen weight (mg)	p value
		Plasmid	Dose, route					
A	5	-	-	-	-	0/5	100 ± 9	-
B	19	pJW	1 µg, id	-	+	19/19	877 ± 523	-
C	10	pJW/gag prot	1 µg, id	-	+	10/10	1719 ± 934	.0263[a]
D	10	pJW/env	1 µg, id	-	+	10/10	2209 ± 1277	.0101[a]
E	10	Combination[1]	1 µg[2], id	-	+	10/10	2076 ± 1281	.0180[a]
F	10	Combination	10 ng, id	-	+	10/10	2187 ± 1401	.0206[a]
G	10	Combination	100 ng, id	-	+	10/10	1187 ± 863	.5715[a]
H	10	Combination	10 µg, id	-	+	10/10	1418 ± 1306	.7389[a]
I	10	Combination	100 µg, id	-	+	10/10	1436 ± 1157	.3376[a]
J	10	Combination	100 µg, id	+	+	10/10	1864 ± 1149	.3643[b]
K	10	pJW	1 µg + boost[3], id	-	+	10/10	1126 ± 1017	-
L	10	Combination	1 µg + boost, id	-	+	10/10	1357 ± 1145	.6831[c]
M	10	pJW	100 µg, im	-	+	10/10	882 ± 795	-
N	10	Combination	100 µg, im	-	+	10/10	2131 ± 1163	.0305[d]
O	10	Combination	100 µg, im	+	+	10/10	887 ± 832	.0161[e]

Legend, Table II

Abbreviation:
IB: immunoblot;
id: intradermal;
im: intramuscular;
iv: intravenous.

[1] pJW/env and pJW/gag prot
[2] 1 μg each
[3] Second DNA
[a] Compared to Group B
[b] Compared to Group I
[c] Compared to Group K
[d] Compared to Group M
[e] Compared to Group N

Time course of DNA vaccination:

9/10/96	d0	DNA, id
9/10/96	d0-4	IL-12, 1 μg/mouse/day, ip
9/24/96	d14	Second DNA, id (if it is needed)
9/30/96	d20	RLV, 20 AID_{50}, 0.2 ml, iv
10/21/96	d41	Collect the sera for IB
10/31/96	d51	Sacrifice mice and weigh the spleens.

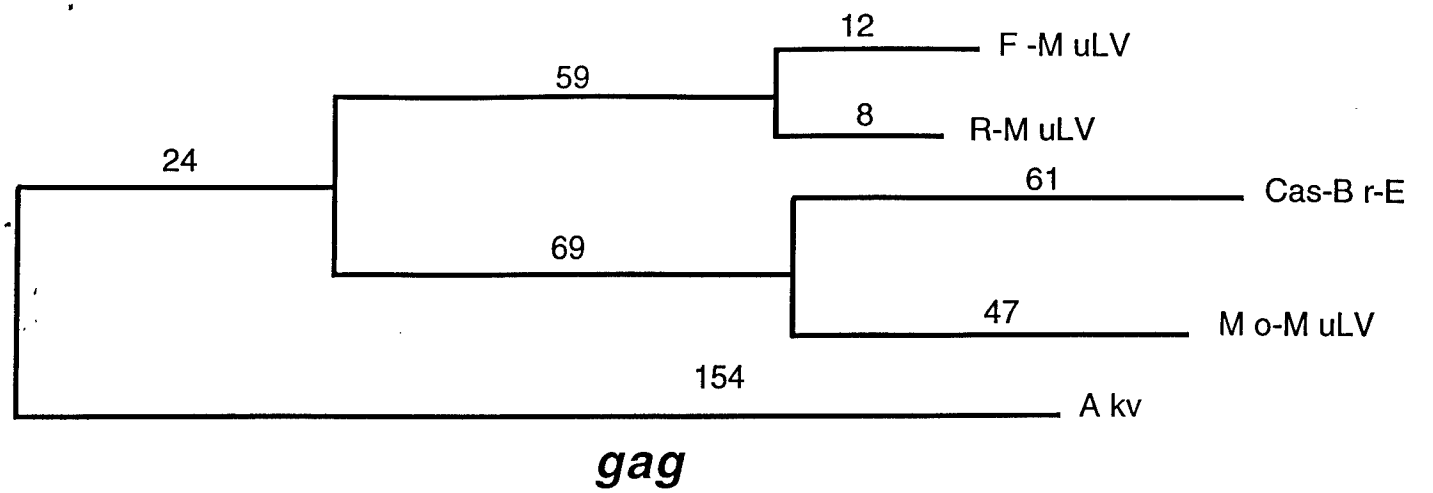
Table III. DNA Vaccines in Neonatal Macaques with IL-12 as Adjuvant: Antibody responses on serum samples of 9/20/96.

Group #	Animal #	DNA	Delivery	IL-12	Positive reaction by WB
A (1)	REu5	5 DNAs	ID	none	
	RIv5	"	"	"	Env, including gp41
	RJu5	"	"	"	Gag
	RUv5	"	"	"	
B (2)	(RSu5) died 7/7/96	5 DNAs	ID	+	N/A
	RTv5	"	"	+	Gag
	RDv5	"	"	+	
	RYv5	"	"	+	
	RMw5 (replacement for RSu5)	"	"	+	Env
C (3)	RWt5	*gag-pol/nef* DNAs	ID	none	Gag
	RBv5	"	"	"	
	ROu5	"	"	"	
	ROv5	"	"	"	
D (4)	RTu5	*gag-pol/nef* DNAs	ID	+	Gag
	RJv5	"	"	+	
	RSv5	"	"	+	
	RCw5	"	"	+	
E (5)	RIu5	control DNA	ID	none	
	RVv5	"	"	"	
	RWu5	"	"	"	
	RZu5	"	"	"	
F (6)	RAw5	5 DNAs	gene gun	none	
	RBw5	"	"	"	Gag and Env (weak)
	RDw5	"	"	"	
	REw5	"	"	"	Gag and Env (weak)
G (7)	RFw5	5 DNAs	gene gun	+	
	RGw5	"	"	+	
	RIw5	"	"	+	Env
	RJw5	"	"	+	

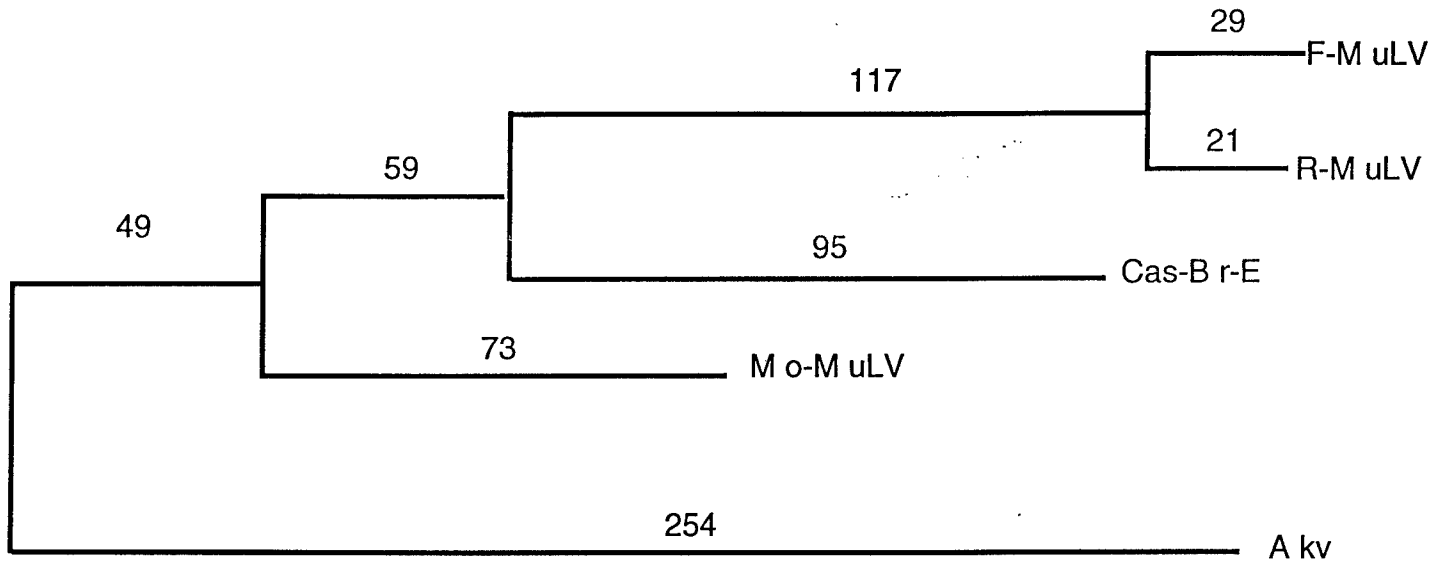
Legend Table III. DNA Vaccines in Neonatal Macaques with IL-12 as Adjuvant: Antibody responses on serum samples of 9/20/96.

Abbreviations: ID, intradermal; gene gun, intradermal delivery via gene gun;
WB, Western blot.

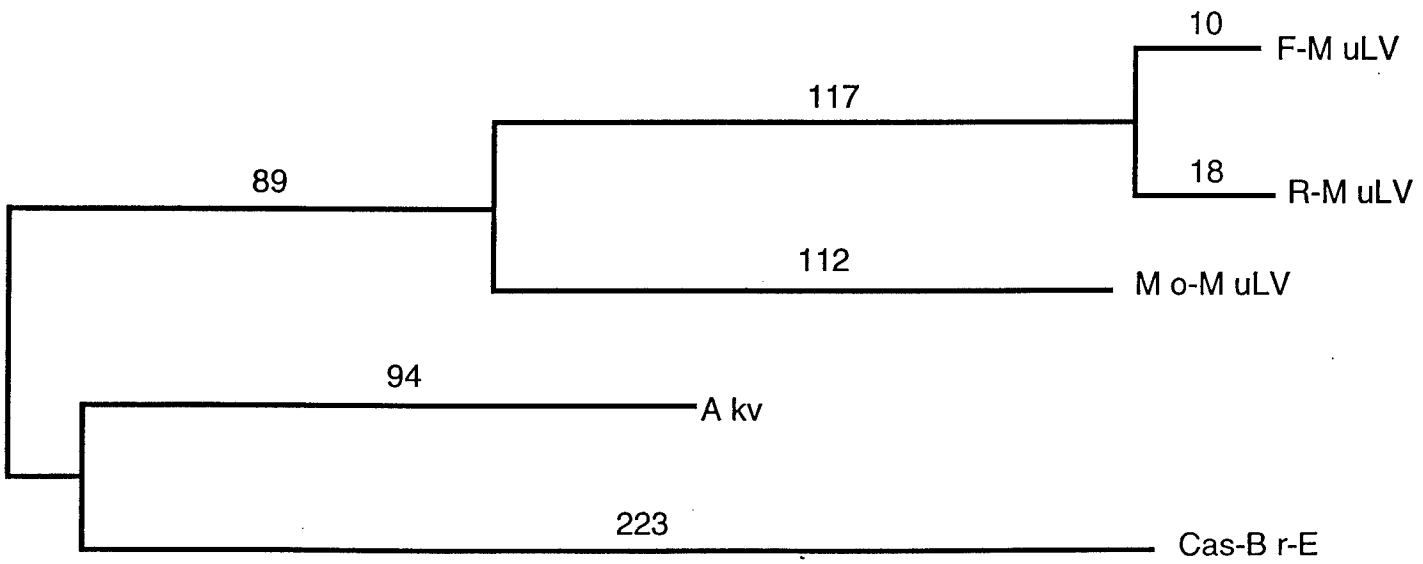
Fig.1



gag



pol

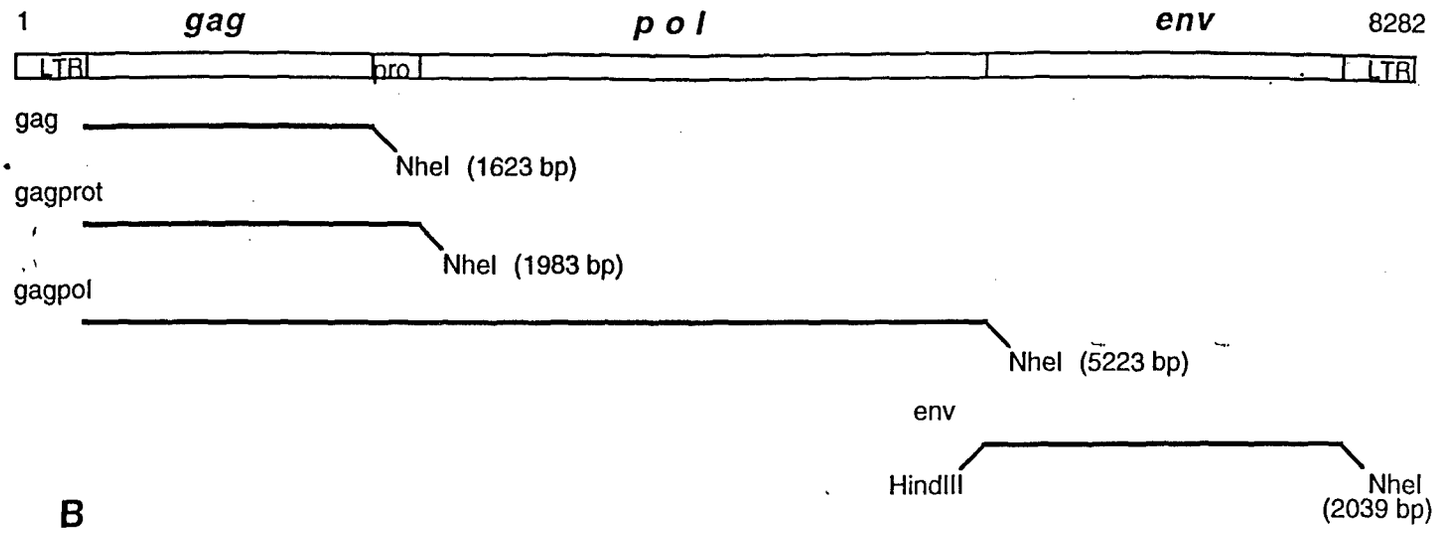


env

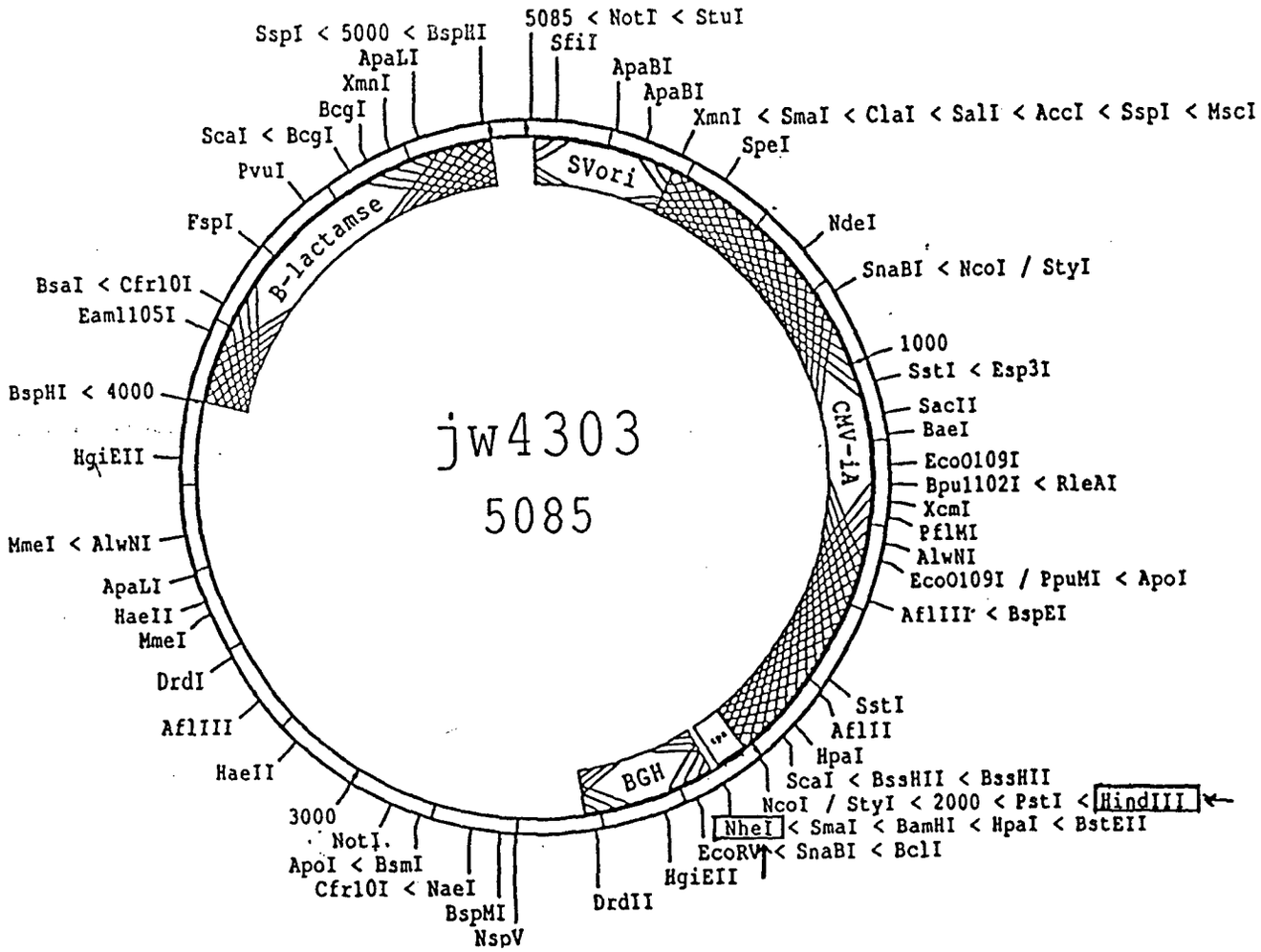
Legend FIG. 1. Phylogenetic relationship of murine retroviruses based on *gag, pol* and *env* sequences. The sequences for each virus were obtained from the Genbank by the accession numbers listed in Table I. The phylogenetic analysis was carried out by initial amino acid alignment of the respective proteins using PIMA (1), followed by tree construction based on codon position 3, using the PAUP software (2). Horizontal branch lengths are indicated above each branch.

Fig.2

A

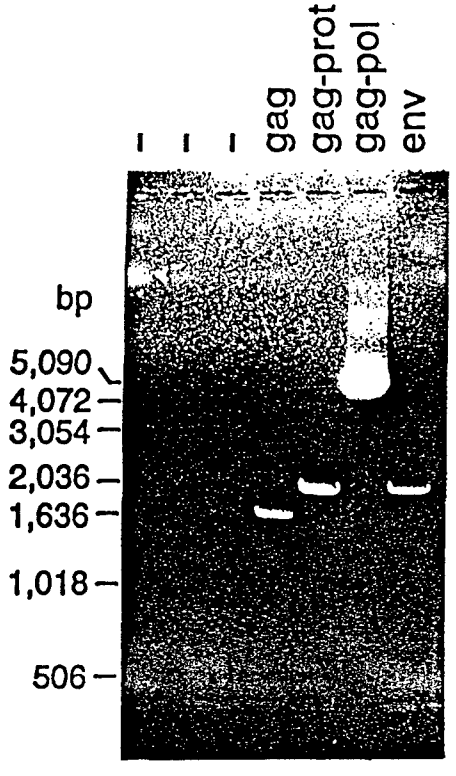

1
gag
pol
env
8282
LTR
pro
LTR
gag
NheI (1623 bp)
gagprot
NheI (1983 bp)
gagpol
NheI (5223 bp)
env
HindIII
NheI
(2039 bp)

B


jw4303
5085
SspI < 5000 < BspHI
5085 < NotI < StuI
SfiI
ApaLI
ApaBI
XmnI
ApaBI
BcgI
XmnI < SmaI < ClaI < SalI < AccI < SspI < MscI
ScaI < BcgI
SpeI
PvuI
SVori
FspI
NdeI
B-lactamse
SnaBI < NcoI / StyI
BsaI < Cfr10I
Eam1105I
1000
SstI < Esp3I
BspHI < 4000
SacII
BaeI
CMV-IA
HgiEII
Eco0109I
Bpu1102I < RleAI
XcmI
PflMI
MmeI < AlwNI
AlwNI
Eco0109I / PpuMI < ApoI
ApaLI
AflIII < BspEI
HaeII
MmeI
DrdI
SstI
AflIII
AflII
HaeII
HpaI
BGH
ScaI < BssHII < BssHII
NcoI / StyI < 2000 < PstI < HindIII
3000
NheI < SmaI < BamHI < HpaI < BstEII
NotI
EcoRV < SnaBI < BclI
ApoI < BsmI
HgiEII
Cfr10I < NaeI
DrdII
BspMI
NspV

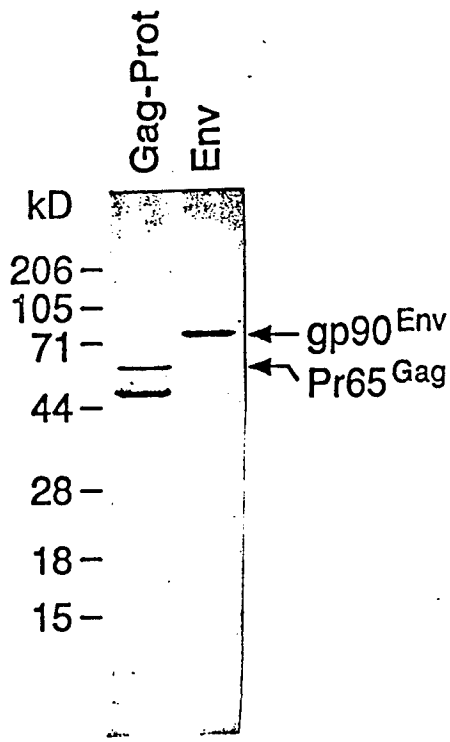
Legend FIG. 2. A schematic representation of the complete R-MuLV genome and the PCR-amplified regions of the genome (*gag*, *gag-prot*, *gag-pol*, and *env*) with expected amplicon sizes (A) is shown. The amplified DNAs were cloned into the *Hind* III and *Nhe* I sites (shown by arrows) of the expression vector, pJW4303 (B).

Fig.3



Legend FIG. 3. The amplified DNA of different regions of the R-MuLV genome (Fig. 2A) were electrophoresed in a 0.8% agarose gel. The negative control reactions with only reagents and primers (without templates) are shown (-), and the individual amplified genes are *gag* (1623 bp), *gag-prot* (1983 bp), *gag-pol* (5223 bp), and *env* (2039 bp) are shown. Molecular size DNA ladders (1 kb) from GIBCO-BRL (position of bands shown on the left) were used as markers.

Fig. 4



Legend FIG. 4. Detection of transiently expressed Gag-Prot and Env proteins by immunoprecipitation with anti-Gag (mAb-34) and anti-Env (mAb-48) from whole cell extracts followed by 10% polyacrylamide gel electrophoresis and Western blotting with anti-RLV polyclonal antibody. The Gag precursor ($Pr65^{Gag}$, shown by arrow) and a cleavage product, and the Env ($gp90^{Env}$, shown by arrow) were detected. Pre-stained molecular weight markers were run, and the relative sizes are shown on the left.

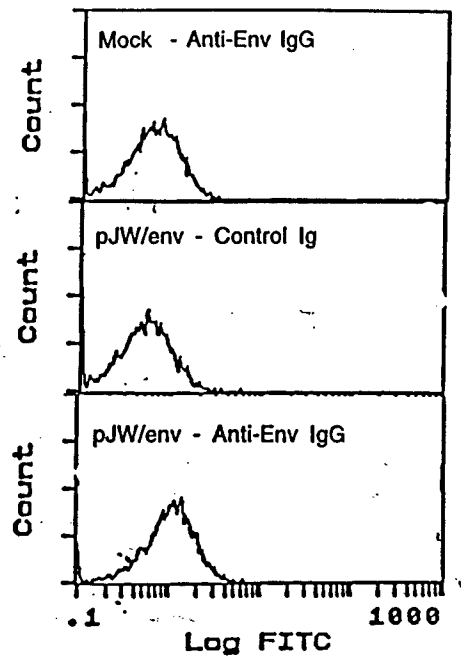


Figure 5. Flow cytometric analysis of P815 stable transfectants. P815 cells, either mock transfected with Rsv-2^{neo} or cotransfected with Rsv-2^{neo} plus pJW/env were stained using either anti-Env IgG or control Ig.

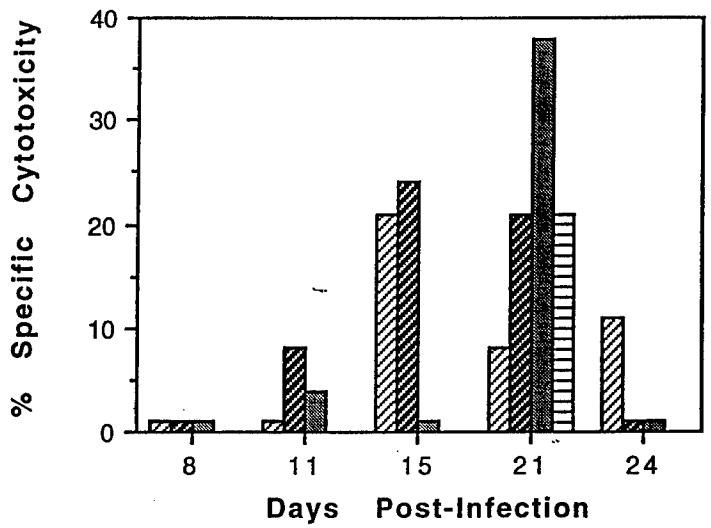


Figure 6. *Ex vivo* RLV-specific CTL activity in AZT plus IFN-α treated, RLV-inoculated mice. Animals were given RLV inoculations (5000 AID_{50}) and started on combination anti-viral drug regimen. On the days indicated, mice were sacrificed and the CTL activity in purified spleen cells was measured immediately without stimulation or culture using Cr^{51}-labeled RLV-infected target cells at an effector:target ratio of 200:1 The results show the CTL response from individual mice at each time point. Killing of control uninfected P815 target cells was always <3% at the same effector to target ratio.

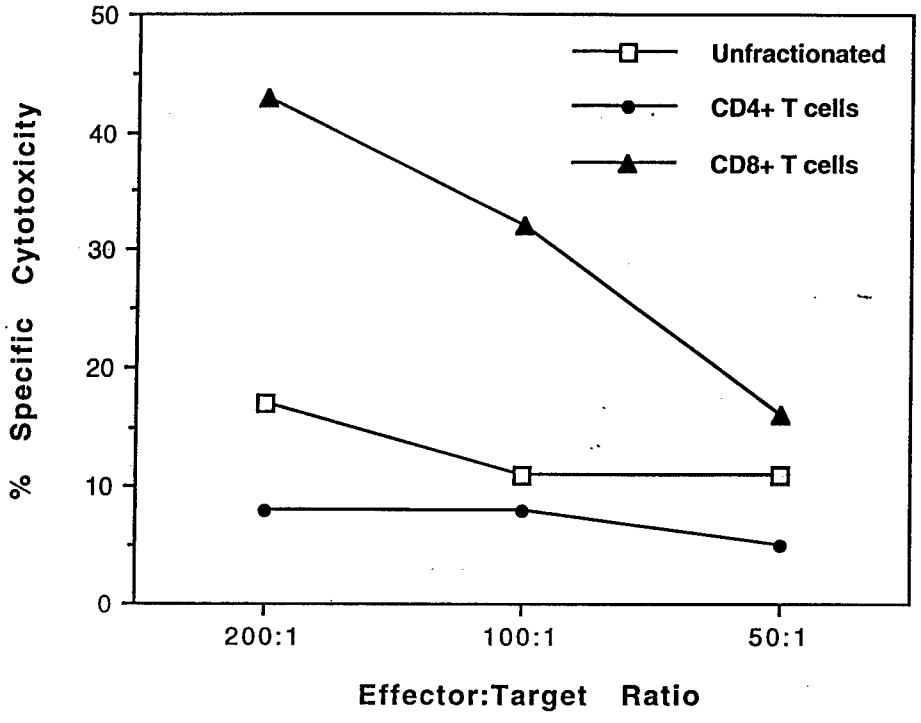


Figure 7. RLV-specific CTL activity of purified CD4+ and CD8+ T cells from AZT plus IFN-α treated, RLV-inoculated mice. Separate populations of CD4+ and CD8+ T cells were isolated by positive selection over immunoaffinity columns from spleens of mice 21 d after RLV inoculation and the start of combination drug therapy. CTL activity against P815-R was then measured for each cell population at the indicated effector to target ratios. There was no CTL activity against noninfected P815 cells.

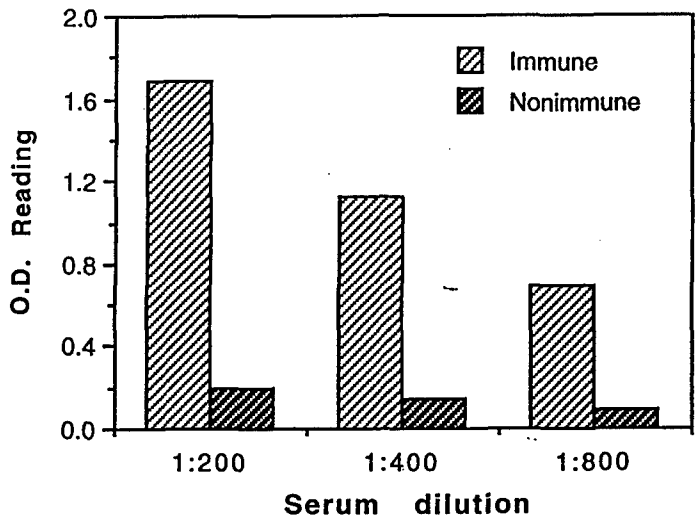
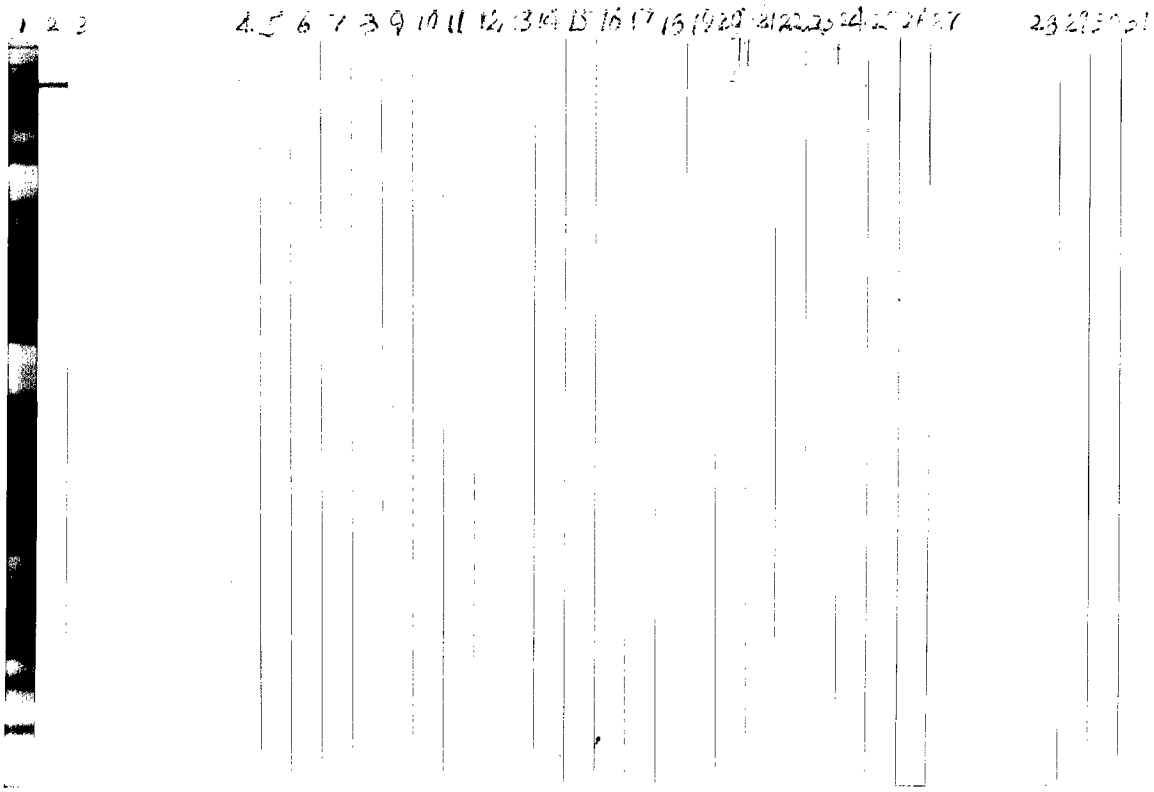


Figure 8. RLV-specific antibody activity in RLV-immunized mice. Serum from mice inoculated with RLV and given combination antiviral drug therepy was collected 21 d post inoculation and tested for RLV-specific antibodies using RLV-infected P815 cells in an ELISA assay. Bound antibodies were detected using peroxidase-conjugated rabbit anti-mouse Ig. Nonimmune serum from mice receiving anti-viral drug therapy alone is shown for comparison.

Figure 9

WESTERN BLOT ASSAY FOR DETECTION OF ANTIBODIES OF #529 TO HIV-1



As the Western blot analysis was carried out blinded, the samples are distributed randomly. For easier interpretation, the experimental groups is given of each animal in parentheses.

1.	strong positive control	12.	RBv5 (C)	22.	RAw5 (F)
2.	weak positive control	13.	RDv5 (B)	23.	RBw5 (F)
3.	negative control	14.	RIv5 (A)	24.	RCw5 (D)
4.	RWt5 (C)	15.	RJv5 (D)	25.	RDw5 (F)
5.	REu5 (A)	16.	ROv5 (C)	26.	REw5 (F)
6.	RIu5 (E)	17.	RSv5 (D)	27.	RFw5 (G)
7.	RJu5 (A)	18.	RTv5 (B)	28.	RGw5 (G)
8.	ROu5 (C)	19.	RUv5 (A)	29.	RIw5 (G)
9.	RTu5 (D)	20.	RVv5 (E)	30.	RJw5 (G)
10.	RWu5 (E)	21.	RYv5 (B)	31.	RMw5 (B)
11.	RZu5 (E)				

* anti-monkey IgG: 1:200 dilutions

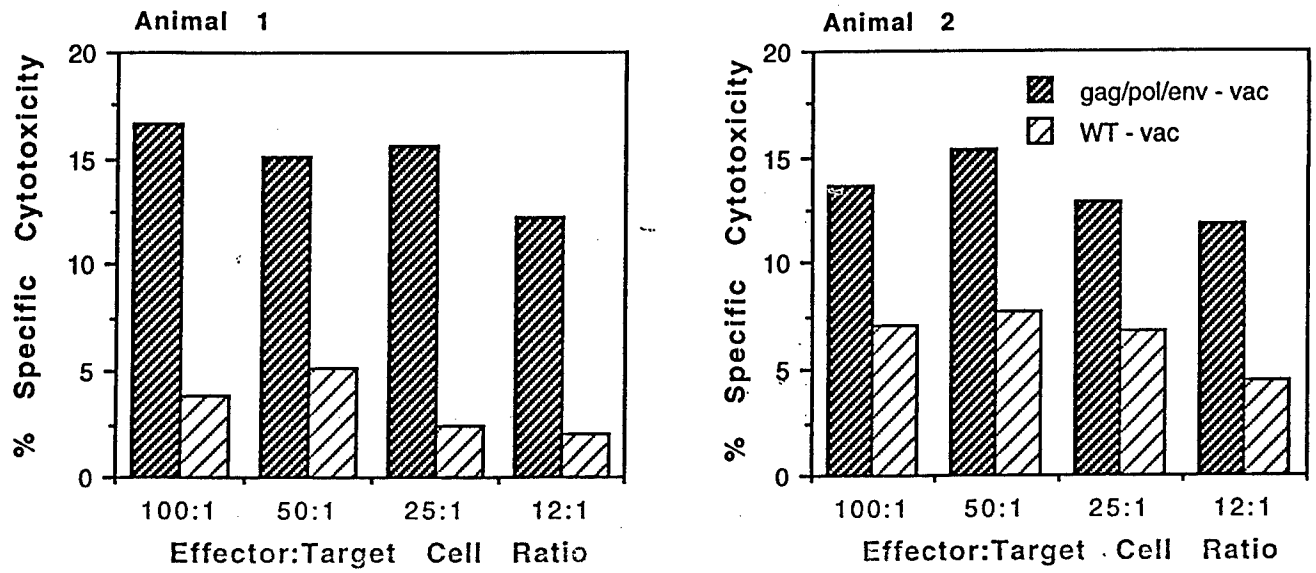


Figure 10. CTL activity in SIVmac251-infected macaques. Peripheral blood mononuclear cells were isolated from two SIVmac251-infected macaques, stimulated with Concanavalin A (4 μg/ml) and maintained in culture with rIL-2 (20 U/ml). After 6 days, activated cells were tested for CTL activity against Cr^{51}-labeled autologous B lymphoblastoid cell lines infected either with wild type vaccinia (WT-vac) or SIV gag,pol, and env-expressing vaccinia (gag/pol/env - vac) at the indicated effector to target ratios. Percent specific lysis of target cells was measured 5 hrs later.

APPENDIX

Attenuated Retrovirus Vaccines and AIDS

In their recent report, Timothy W. Baba *et al.* state that a deletion mutant of simian immunodeficiency virus (SIVΔ3), which does not cause disease in adult macaques and has been successfully used as a vaccine against challenge with pathogenic virus (*1*), causes acquired immunodeficiency syndrome (AIDS) in newborn macaques. They ascribe the differential outcome of SIVΔ3 infection of neonatal and adult macaques to several possibilities including the amount of virus replication early after inoculation, the route of virus inoculation, and the developing neonatal immune system. However, their study does not allow separation of these important variables.

We found that high-dose intravenous inoculation of newborn rhesus macaques with molecularly cloned SIVmac239 (the parental virus from which SIVΔ3 was derived) resulted in persistently high amounts of virus in peripheral blood mononuclear cells (PBMC) and plasma (higher than those reported by Baba *et al.* for SIVΔ3). Rhesus newborns infected with SIVmac239 did not experience rapid $CD4^+$ T lymphocyte depletion, and the time course before fatal immunodeficiency developed was consistent with that previously reported for SIVmac239-infected adult macaques (that is, 6 to 24 months) (*2, 3*). Thus, an age-related difference does not explain why rhesus infants inoculated with an attenuated triple-deletion mutant of SIVmac239 appear to experience a more rapid $CD4^+$ T cell depletion and $CD4^+/CD8^+$ T cell ratio inversion than rhesus infants inoculated with the pathogenic parental virus, SIVmac239. We also found that absolute $CD4^+$ T lymphocyte numbers were not a reliable marker of disease progression in infant rhesus macaques because of extreme variability of absolute lymphocyte counts in response to stress (for example, handling). Only absolute $CD4^+$ T cell numbers that are persistently below 500 per microliter reliably suggested $CD4^+$ T lymphocyte depletion in neonatal macaques (*2–4*).

Baba *et al.* hypothesize that the oral route of inoculation may be responsible for increased virulence of SIVΔ3 in newborns. Our observations with five orally and six intravenously inoculated newborn macaques did not demonstrate a more severe course of infection with uncloned pathogenic SIVmac251 for the oral route (*2–4*). With regard to the postulated age-dependence of SIV virulence, we have also compared the time course of infection of the nonpathogenic molecular clone, SIVmac1A11, and SIV/human immunodeficiency virus–1 (HIV-1) envelope chimeric viruses in macaques of different ages: We have no evidence that an SIV strain that is attenuated in older macaques becomes pathogenic when inoculated intravenously or orally into newborn macaques (*2, 5*). Instead, inoculation of fetal and newborn macaques with attenuated SIVmac1A11 proved to be a safe and effective vaccine against challenge with pathogenic uncloned SIVmac later in life (*3*). Finally, our studies indicate that the neonatal immune system was not overwhelmed by attenuated SIV isolates or by a pathogenic SIV clone (*2, 3*).

Caution must be used when assigning the underlying cause of death in SIV-infected macaques to immunodeficiency. For the one SIVΔ3-inoculated macaque that died in their study, the classical hallmarks of simian AIDS (such as the presence of opportunistic infections, encephalopathy, and so on) apparently were not demonstrated by Baba *et al.* Instead, this animal had severe anemia and thrombocytopenia, reportedly a result of peripheral autoimmune destruction of red blood cells and platelets. It is not clear whether this diagnosis of hemolytic anemia was mainly based on a positive direct Coombs test. Many healthy macaques will react positively if human Coombs test reagents are used (*6*). Clinical hemolytic anemia must be confirmed by additional evidence, such as hemoglobinuria, poikilocytosis, the presence of spherocytes, hemolytic or icteric plasma, and increased serum bilirubin and lactate dehydrogenase. The erythroid hyperplasia of the bone marrow, reported by Baba *et al.*, is a finding that we do not see in anemic SIV-infected animals; rather, their bone marrow aspirates reveal a myeloid hyperplasia with the erythroid series being normal or only slightly increased (*7*). Findings in addition to an abundance of megakaryocytes in the bone marrow are needed to support the hypothesis of peripheral platelet destruction. SIV-infected animals often have a megakaryocyte hyperplasia of the bone marrow, but these megakaryocytes have increased cytoplasmic vacuolization, which suggests that the thrombocytopenia is a result of decreased platelet production rather than peripheral platelet destruction (*6*).

Extra care needs to be taken to exclude all other pathogens that can adversely affect the immune system and the health of macaques. Although the animals in the study by Baba *et al.* were polymerase chain reaction–negative (by an assay able to detect approximately one infected cell in 8000 PBMC) and seronegative for simian type D retroviruses, virus isolation is more reliable for diagnosis of this viral infection, but was not reported by Baba *et al.*

Until a more thorough analysis is completed and results of Baba *et al.* are confirmed, it would be premature to dismiss the potential of SIV *nef*-deletion mutants as live-attenuated vaccines.

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The report by Baba *et al.* raises concern that attenuated HIV may not be safe as a vaccine to prevent syndrome AIDS. A major point of the report is that mucosal infection of newborn macaques with a triple gene-deleted preparation of attenuated SIV can result in an AIDS-like condition with a reduction in the CD4 cell count. This observation contrasts with the experience of Desrosiers and his colleagues who observed no ill effects in adult macaques that had been infected with a triple deletion SIV (*1*). Although the differences observed in the two studies might be accounted for by differences in the doses and routes of attenuated SIV administered, as noted by Baba *et al.*, an important host factor that needs to be considered is the difference in the strength and maturation of the immune systems of neonates and adult animals.

The immune response potentials of adult and neonate macaques are likely to be different, such that antigen-presenting cell (APC) function for generating strong cellular immune responses could be deficient in the neonates, as they are in healthy human infants younger than 1 year of age (*2*). This type of deficiency could result in an inability or reduced ability of the cellular arm of the immune system to control the extent of replication of the attenuated virus. In contrast, the competent immune system of the adult animals would be expected to hold the attenuated SIV in check, and could result in protection against challenge with wild-type SIV. Such a defect in the APC function of neonates could permit viral replication and the generation of viral products that might be responsible for $CD4^+$ T cell depletion. It has been reported that after priming in the presence of interleukin-12, human naïve neonatal $CD4^+$ T cells appear to develop a T helper cell zero (T_H0) phenotype, whereas adult naïve $CD4^+$ T cells develop into T_H1 cells (*3*). If this difference exists in macaques as well, it could contribute to differences in the immune potential between neonates and adults.

Concerning safety in the use of these attenuated viruses as vaccines, it might be argued that an attenuated HIV vaccine would be safe to use in adults whose immune systems are adequate to control a gene-deleted virus. However, the question remains as to whether the immune system of adults would continue to be sufficiently competent to hold the attenuated virus in check. Other infections, immune-suppressive drug therapy for other conditions, and aging could render the immune system inadequate to control the attenuated virus. Therefore, it is important to determine whether adult macaques that have been infected with attenuated SIV for an extended time will continue to maintain normal CD4 counts and remain without symptoms after immune suppression is induced.

Finally, central nervous system damage poses an additional safety issue for HIV vaccines that may be particularly relevant for infants. HIV-1 is frequently associated with neurologic and behavioral conditions in infants and children, in whom the virus may infect astrocytes as well as microglia, with the *nef* gene implicated in contributing to neuropathologic damage (*4*). Thus, it would be of value to have assessed neuropathologic and neurovirologic parameters at autopsy in the macaques receiving the *nef*, *vpr*, NRE-deleted live SIV vaccine.

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If SIVΔ3 is pathogenic in macaque infants (*1*, *2*) then, by analogy, HIV with deleted genes might be pathogenic in human infants. Infants must not, therefore, be vaccinated or exposed to these potentially pathogenic HIV mutants. As there are no data thus far that *nef*-deleted SIV is pathogenic in adult macaques, the findings of Baba *et al.* only preclude the vaccination of women who might infect their infants or neonates with HIV having deleted genes. The solution may be to immunize immunocompetent men only. A strategy using an effective attenuated HIV vaccine in men could stop the spread of HIV. This strategy would be effective because men infect men, and men also infect women (*3*). Women do not infect women to any degree (*3*), if at all, and men do not infect infants or children under normal circumstances. Finally, women would not transmit HIV to immunized men. The cycle would be effectively broken. Eventually, all new infections except needle-transmitted HIV between women would be stopped by immunizing men only.

It must still be determined if immunized men would shed enough virus to transmit the vaccine virus to women. Transmission of the vaccine strain between men would not be a problem, as any given two men would be immune in this protocol. Male macaques and SIVΔ3 could be used to test this approach to vaccination.

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We have reported on the development of a second generation live *nef* attenuated vaccine strain (*1*) based on a gain-of-function approach that could address the safety issues raised by Baba *et al.* This concept is exemplified by the addition of a conditionally lethal suicide gene to a *nef*-deleted (loss-of-function) vaccine strain. Preliminary results suggest that the gain of a conditionally lethal function on top of a loss of a critical virus gene for growth further reduces virus load and perhaps affords greater safety. While we have worked on one prototypic effector gene [herpes simplex virus–1 (HSV-1) thymidine kinase], we realize that many other effectors are possible in attenuating a live vaccine through gain-of-function. Issues concerning a neutral or even a positive selective force needed to maintain a gain-of-function must be addressed before this approach is feasible. Genetic strategies for accomplishing this selection exist, and future refinements on a gain-of-function approach are likely.

Gain-of-function should be considered with loss-of-function in designing safe live attenuated HIV-1 vaccines.

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Response: We demonstrated that SIVΔ3, a mutant of the SIV deleted in the *nef* and *vpr* genes, induced lethal AIDS in two of four macaque neonates infected orally, but remained attenuated in the adult after intravenous (iv) infection (*1*). Persistently high virus loads were seen in infants, in contrast to adults who seroconverted and were culture-positive only in the first few weeks after inoculation (*1*, *2*). To explain this differential pattern of viremia and pathogenicity, we introduced two new concepts: The threshold hypothesis and a working definition for retrovirus attenuation (*1*, *3*).

According to the threshold hypothesis, retroviral pathogenicity can only become apparent after the virus replicates above a threshold in a given host (Fig. 1). If virus replication is repressed by any mechanism or mechanisms, disease will not ensue, *even if the virus contains the gene or genes that encode virulence*. We postulate that, in macaque neonates, replication of SIVΔ3 was unrestricted and exceeded the threshold, whereas host factors limited replication in adults (*1*, *2*). Although the mechanisms for the differential pathogenicity of SIVΔ3 in adults versus neonates remain to be determined, we agree with Shearer *et al.* that decreased cellular immune responsiveness in neonates may play a major role. Alternatively, activation of the neonatal immune system by encounters with environmental antigens could favor the replication of a *nef*-deleted virus. In vitro studies have shown that Nef augments HIV-1 replication in primary unstimulated T cells, but has no effect in stimulated T cells or permanent T cell lines (*4*).

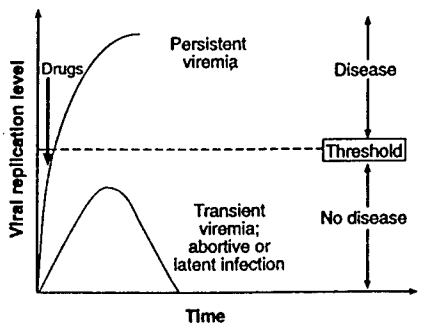

Fig. 1. Host-retrovirus interactions. Development of disease after infection with a retrovirus occurs only after the level of virus replication has exceeded the threshold. Even a fully pathogenic virus can exhibit a pattern of transient viremia and abortive or latent infection if the level of replication does not reach the threshold. Host defenses, antiviral drugs (arrow), or immunotherapy could be used to restrict virus replication soon after infection.

We propose to classify attenuated viruses into two broad categories according to the *mechanism of attenuation*: Replication-impaired viruses and avirulent viruses, which would not cause disease even in immunocompromised hosts, regardless of their replicative capacity (Table 1). Factors that upregulate virus replication could compensate for the relative loss of replicative power in replication-impaired retroviruses, and virulence could be restored once the threshold is exceeded. Attenuated viruses may also exhibit a mixed pattern within a wide spectrum of partial replication impairment and partial loss of pathogenicity. As SIVΔ3 caused AIDS in macaque neonates, *nef* is not the major molecular determinant for virulence; rather, this gene modulates virus load and influences pathogenicity only indirectly. Other studies demonstrated that the *vpr* gene does not control virulence either (*5*, *6*). Consequently, SIVΔ3 is classified as a replication-impaired rather than avirulent virus.

Other data support the threshold hypothesis. In SCID (severe combined immunodeficiency disease) mice with human fetal thymus transplants, *nef*$^+$ HIV-1 replicated and severely depleted thymocytes within 6 weeks, whereas a similar inoculum of *nef*-deleted HIV-1 revealed little cytopathicity (*7*). However, a tenfold higher inoculum of *nef*-deleted virus induced thymocyte loss in a significant fraction of implants by 9 to 12 weeks after infection (*7*).

The threshold hypothesis is confirmed by our studies with Rauscher murine leukemia virus (RLV) (*8*). First, a short course of post-exposure antiviral therapy prevented viremia and disease in all mice inoculated with a high RLV dose, and most resisted rechallenge by the same viral dose without further drug therapy. Thus, pathogenic RLV acted as a pharmacologically attenuated, live virus vaccine (Fig. 1, arrow). Second, normal mice were inoculated with low doses of pathogenic RLV which were still infectious in nude, athymic mice. No antiviral therapy was given. Normal mice without signs of infection were rechallenged with 20 animal infectious doses of RLV; 21% were immune, indicating that protective antiviral immune responses can be generated with a fully pathogenic virus, as long as replication remains below threshold (*9*). Finally, although the pharmacologically attenuated, live RLV vaccine protected many adult mice against high-dose challenge (*8*), the vaccination caused RLV disease when animals were inadvertently co-infected with mouse hepatitis virus (*9*). This experiment reveals a serious danger of vaccine strategies that use replication-impaired live retroviruses: *Success is based on host defenses outracing vaccine virus replication*. Because RLV was only attenuated by drugs interfering with its ability to replicate, the vaccine strategy failed when the animals were temporarily immunocompromised (*9*).

Van Rompay *et al.* discuss SIVmac1A11 (*10*), which has an open *nef* reading frame and several mutations in structural genes which differ from the targeted deletions in SIVΔ3 (*11*). The low virus loads and lack of disease in macaque neonates infected iv with SIVmac1A11 indicate replication impairment and do not contradict our data with *nef*-deleted SIVΔ3, which we classify as replication impaired rather than avirulent. Whether SIVmac1A11 is attenuated also for virulence remains to be determined.

Other issues raised by Van Rompay *et al.* need clarification. They imply that SIVΔ3-infected macaque infants died faster than their infants infected with parental SIVmac239, which died at 34 and at more than 54 weeks of age (*12*). The time to death within our series was 34 weeks and 46 weeks, respectively (*1*), and two infants are still alive at 19 months of age. Van Rompay *et al.* mistakenly infer that we only saw spuriously low CD4$^+$ T lymphocyte counts in our macque infants. It is important to consider that CD4$^+$ T cell counts are considerably higher in normal macaque infants than in adults. In human infants less than 1 year of age, CD4$^+$ T cell counts of less than 1500 per microliter are abnormal (*13*). Infant 93-7 had less than 500 CD4$^+$ T cells per microliter in every assay (*1*), and animal 94-4 had persistently low CD29$^+$CD4$^+$ T cell subsets, inverted CD4$^+$/CD8$^+$ T cell ratios, and 188 CD4$^+$ T cells premortem. Of the two survivors, one has been thrombocytopenic for a year with CD4$^+$ T cell counts that are persistently low for its age, and both have depleted CD29$^+$CD4$^+$ T cell subsets. Although route of exposure to SIVmac239 did not influence virulence in

Table 1. Mechanisms of retroviral attenuation. Theoretically, a retrovirus can be attenuated for its ability to replicate, while retaining the gene or genes encoding pathogenicity. Such a virus can become dangerous if the relative loss of replicative power can be compensated by other factors. An avirulent virus, on the other hand, will not cause disease even if the virus is fully replication competent. Consequently, no disease-defining threshold for the level of viral replication exists for this avirulent virus in the host (parentheses).

Mechanism of attenuation	Virus designation	Replication above threshold		Disease	
		In normal host	In co-infected or immuno-compromised host	In normal host	In co-infected or immuno-compromised host
Decreased ability to replicate	Replication-impaired	No	Yes	No	Yes
Loss of pathogenicity	Avirulent	(Yes)	(Yes)	No	No
None	Pathogenic	Yes	Yes	Yes	Yes

infant macaques, Van Rompay *et al.* did not address the differential pathogenicity of *nef*⁻ mutants in iv infected *adults* and orally infected *infants*. Our preliminary data indicate that age, rather than route of virus exposure, determines SIVΔ3 virulence.

The clinical course and cause of death in our SIVΔ3-infected macaque infants resembled those in macaque infants infected orally with wild-type SIVmac251 (*14*). Typically, these infants do not die of opportunistic infections in our biocontainment facility and do not develop gross neurological dysfunction. The spectrum of disease, that is, immunodeficiency, thrombocytopenia, anemia, and renal disease, is seen also in HIV-1–infected children (*13*). We diagnosed hemolytic anemia in macaque infant 93-7 by persistent reticulocytosis and erythroid hyperplasia in the bone marrow in the absence of overt and occult blood loss, and only confirmatory, by the Coombs test. Large platelets on peripheral blood smears and megakaryocytosis in the bone marrow of the thrombocytopenic infant 93-7 are consistent with peripheral platelet destruction.

We raised the issue of potential adventitious pathogens (*1*); to rule out infection with known simian retroviruses, serial serological testing was performed and diagnostic PCR assays were developed for simian T lymphotropic virus–type I (STLV-I) and simian type D retroviruses (SRV/D). The sensitivity for the latter has been increased; we now detect 0.7 to 1.0 proviral DNA copies of SRV/D serotypes 1, 2, and 3 in 150,000 cells (*15*). Serial samples available from the experimental mother/infant pairs were subjected to a blinded re-analysis; no SRV/D sequences were found. Neither SRV/D isolation, serology, nor PCR analysis are described routinely in the SIV literature. We propose that all macaques be prescreened by SRV/D serology and PCR prior to enrollment into SIV studies.

At first glance, the vaccination strategy suggested by Marx may appear to provide a simple solution; by administering live attenuated HIV-1 to immunocompetent men only, the virus transmission cycle would be broken effectively. We wish to address several potentially serious problems with this strategy.

First, without an animal model for HIV-1 virulence, mutant viruses attenuated for their ability to replicate may have residual virulence that would become manifest only in humans. The relative loss of replicative power of such vaccine viruses could be compensated by various mechanisms, including co-infection with other pathogens, ultraviolet radiation, temporary loss of immunocompetence due to intercurrent illness, or aging. If vaccine virus replication exceeds threshold, AIDS may develop, even in adult men. Safety studies with *nef*-deleted viruses in adult macaques have been limited, and the effects of immunosuppression or immune activation on vaccine virus replication are unknown. Second, the generation of protective responses after vaccination with live attenuated retroviruses depends on adequate levels of replication; if a vaccine virus is too weakened, protection from wild-type pathogenic virus will not be achieved (*16*). Even relatively low levels of viral replication carry the risks of insertional oncogenesis (*17*) and generation of mutant viruses. Disseminated lymphoproliferative disease was reported in an adult macaque 29 months after infection with *nef*-deleted SIV (*18*). Third, all adult rhesus monkeys given *nef*-deleted SIV mutants replicated vaccine virus to high levels for several weeks after vaccination (*1*, *2*). During similar initial peaks of viremia after vaccination with *nef*-deleted HIV-1, virus could be transmitted to women who could pass the infection to their children. Ho and Cao (*19*) reported that a woman, identified as HIV-1-infected because she delivered an infected infant, had no disease for more than 12 years. Even though the virus isolated from this mother was attenuated in cultured cells, her child died at age 12 of AIDS. Possibly, the child's virus evolved from an attenuated to a more virulent form, or alternatively, the "attenuated" HIV-1 was more virulent in the susceptible young host, as we demonstrated in macaques (*1*).

Lastly, protective mechanisms following vaccination of adult macaques with SIVΔ3 are slow to develop (*2*); only animals rechallenged with wild-type virus 79 weeks after vaccine administration were protected, but not those re-challenged at 8 or 20 weeks. Slow development of protective responses after vaccination could be dangerous in human vaccine recipients. A false sense of security could lead to increases in risk behavior, thus increasing the chance of wild-type HIV-1 infection during the long time period required for protective responses to mature.

Could *nef*-deleted lentivirus vaccines be made safer? Kestler and Jeang describe a novel concept to increase vaccine safety; a conditionally lethal suicide gene (gain-of-function) will be cloned into the position of *nef*. While currently no in vivo safety and efficacy data are available to evaluate this approach, *nef*-deleted SIV tends to further delete sequences close to the original deletion (*1*, *20*); selective pressure may need to be exerted to retain a conditionally lethal suicide gene. Whether a generally applicable vaccine strategy can be derived from this approach, using a virus genome with residual virulence, is not known.

In agreement with Shearer *et al.*, we feel it is premature to consider *nef*-deleted viruses safe, even in adults. Many factors could disturb the fine balance between the rate of vaccine virus replication and the ability of host defenses to contain replication; the tug-of-war between these two opposing influences could be decided in favor of virus replication in the presence of other pathogens or disturbances in the host immune system. Meanwhile, research with *nef*-deleted viruses should proceed to determine the correlates of protection. Even if these viruses are unsafe as human anti-AIDS vaccines, important insights into the mechanisms of resistance to infection with wild-type virus can be gained. After the protective principles are identified, safer vaccine strategies can then be tested for their ability to induce the same host responses.

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Murine and simian retrovirus models: the threshold hypothesis

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Introduction

Animal models have played an important role in dissecting the interplay between hosts and pathogenic retroviruses. In most retroviral systems, a clinically silent period of viremia precedes the development of overt disease. Studies of the interaction between HIV-1 and the host have been hampered by the lack of a suitable animal model in which both viremia and disease can be studied. Although HIV-1 can infect chimpanzees [1,2], only one out of over 200 infected animals has developed AIDS [3,4] after more than 10 years of follow-up. Thus, the chimpanzee model remains restricted to the study of viremia.

HIV-1 has been reported to infect pigtailed macaques (*Macaca nemestrina*) [5–9]; however, it is not known whether full-blown AIDS will develop, and thus follow-up studies continue. HIV-2 has been inoculated into various species of macaques [10–14], and so far, disease has been described in pigtailed macaques (*M. nemestrina*) [15]. Baboons are also susceptible to HIV-2 infection [12,16–18], and an AIDS-like illness has been reported [18].

Because no practical small animal system is available that permits the modeling of HIV-1 viremia as well as AIDS, animal models involving surrogate retroviruses have been used. Results from such experimental systems have advanced our understanding of pathogenesis and are important for the development of antiviral drugs and vaccines. The first anti-HIV-1 drug approved for the treatment of human AIDS, 3'-azido-3'-deoxythymidine [zidovudine (ZDV)] [19–22], was tested and found to have antiretroviral activity initially in mice infected with type C retroviruses [23], which lack the regulatory genes characteristic of primate lentiviruses [24,25]. ZDV post-exposure chemoprophylaxis [26] and prevention of maternal–fetal transmission [27–30] were also originally demonstrated in mouse models.

HIV-1 does not infect normal mice, but C.B.17 mice with a genetically defined severe combined immunodeficiency syndrome (SCID) accept human fetal tissue transplants (SCID-hu mice) [31] or peripheral blood mononuclear cells (PBL) from adult humans (hu-PBL-SCID mice) [32]. Human tissues and cells in these chimeric mice support HIV-1 replication [33–36].

The discovery of the simian immunodeficiency virus (SIV) [37] has led to major advances in AIDS research. Because the SIV genome is related to HIV-1 [38] and the clinical disease spectrum in macaques mimics human AIDS [39,40], the SIV–macaque model is widely considered to be the most relevant model system [41,42]. This animal model has already played a key role in defining pathogenesis and developing vaccines against AIDS.

Success of post-exposure chemoprophylaxis depends on host cellular immunity

We have focused on the interplay between retroviruses and their hosts, which has led us to rely on animal models. Our earliest studies involved Rauscher murine leuke-

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mia virus (RLV) infection of mice. After a relatively short period of viremia (2 weeks), infected mice develop splenomegaly, followed by frank erythroleukemia a few weeks later [43]. We have chosen this model system because the degree of splenomegaly measured 3 weeks post-inoculation is proportional to the initial virus inoculum, and second, because infectious virus can be quantitated by plaque assay. Among various cell types, RLV also targets dendritic cells early on and inhibits their function, thus mimicking an important aspect of HIV-1 cell tropism [44].

We used this practical murine model system to test whether post-exposure prophylaxis with an antiviral agent blocking early steps in the viral life cycle could prevent viremia and disease. A 3-week treatment course of ZDV or ZDV combined with interferon-α inhibited the development of chronic viremia and disease [26,45]. After cessation of therapy, no infectious virus was found in hundreds of RLV-exposed, drug-treated mice tested. Chronic viremia was still preventable, even though therapy was not initiated until 96 h post-inoculation [46]. Since the retroviral life cycle is completed in approximately 12 h, several full rounds of RLV replication must have taken place before the onset of drug therapy. This implied that the host was able to ward off a low level of replication, provided further virus spread was blocked by drugs.

To test whether host immunity was involved in the success of drug prophylaxis, post-exposure prophylaxis was evaluated in parallel in normal BALB/c mice and in their nude, athymic counterparts [47]. At the standard RLV inoculum, viremia was prevented only in normal mice. Drug prophylaxis protected most nude mice only when a 10-fold lower virus dose was given. These results revealed that host cellular immunity contributes significantly to the success of drug prophylaxis.

The nature of the host immune responses was evaluated as follows. One group of mice was inoculated with RLV, and the control group was given normal saline at time zero [45]. Both groups were treated with ZDV plus interferon-α combination therapy for 20 days. Approximately 1 week after cessation of therapy, both groups of mice were challenged with high-titer, live RLV. Mice exposed previously to virus did not develop viremia or disease. In contrast, all drug-treated control mice became infected and died. Surprisingly, exposure to RLV under drug coverage had converted live, fully pathogenic RLV into a vaccine. This unconventional 'vaccine' virus was attenuated by drugs that interfered with the viral replication cycle. Subsequently, we determined the correlates of immune protection by adoptive transfer. Naive recipient mice could be protected against RLV challenge by immune T cells alone [46]; both immune CD4+ and CD8+ T-cell subsets were required [48]. No neutralizing antibodies were detected in the vaccinated mice [46].

To test whether the drug blockade was complete or partial in the virus-exposed animals, mice were sacrificed at regular, short time intervals during post-exposure prophylaxis, and their spleens were analyzed by polymerase chain reaction (PCR) for the presence of proviral sequences. In virus-exposed control mice, faint proviral signals were found immediately after inoculation, which increased in intensity from day 4 onward and became prominent by day 20. In virus-exposed drug-treated animals, the intensity of the proviral signals increased also up to day 14, at which point it started to diminish. At the end of therapy on day 20, proviral sequences could no longer be detected in many drug-treated mice (unpublished data). Clearly, the virus replicated at low levels despite the presence of drugs. We hypothesize that cellular host immune responses were responsible for the elimination of infected cells. On the basis of these initial observations, as well as data obtained later in our primate studies, we formulated the threshold hypothesis.

The threshold hypothesis

The basic tenet of the threshold hypothesis holds that in every person or animal, old or young, the pathogenicity of a retrovirus will only become apparent after a certain threshold of viral replication has been exceeded (Fig. 1) [49–51]. If the viral load remains below threshold, a retrovirus will not cause disease, even if it encodes the gene(s) that determine virulence. Subthreshold levels of virus replication could have several different outcomes. Host cellular immunity could eliminate infected cells, leading to transient or abortive infection. Alternatively, the virus could become latent. Should the integrated provirus become activated at any time later, host immune surveillance would keep the level of replication in check. Finally, subthreshold replication could also result in persistent, smoldering infection with low virus loads.

The threshold effect makes it necessary to give a clear definition of viral attenuation, because even fully pathogenic viruses can appear 'attenuated' if the viral load does not exceed threshold. We have proposed to classify attenuated retroviruses according to the mechanism of attenuation (Table 1). To conceptualize, we have assumed that the mechanics of virus replication can be dissociated from the capacity of the virus to cause disease. If a virus is weakened in its ability to replicate, it may appear to have lost virulence, solely because the threshold cannot be reached in a normal host. Such viruses are designated as replication impaired. Theoretically, viruses could be mutated or deleted in the gene(s) encoding virulence while maintaining their ability to replicate well. Such viruses are termed avirulent.

Because most retroviral infections are life long, the balance between host and virus can shift with time in favor of the virus. Host immunosuppression or upregulation of virus replication by various mechanisms, including trans-

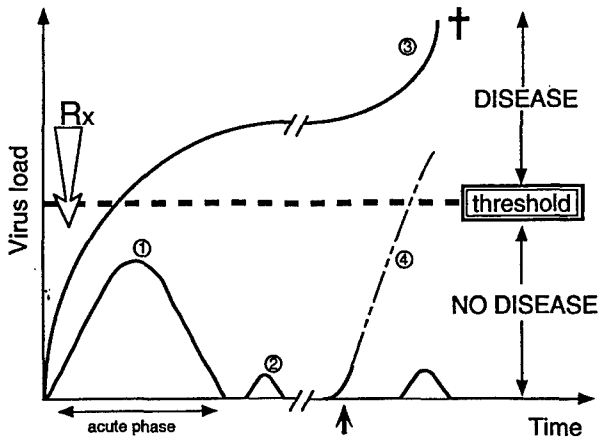

Fig. 1. The threshold determines long-term clinical outcome after acute viral exposure. If viral loads do not reach threshold during the acute phase (1), disease will not develop, even if the virus has the genes that determine virulence. Instead, the result could be elimination of all traces of infection, latency, or smoldering low-grade infection (2) characteristic of the pattern seen in some long-term non-progressors with HIV-1 infection. If, on the other hand, the threshold is exceeded during the acute phase, disease will develop (3). Hypothetically, an individual with subthreshold smoldering infection could become immunosuppressed or coinfected with other pathogens ↑. Such events could upregulate virus replication to exceed threshold (4). Disease could then develop. The race between the virus and the host could be decided in favor of the latter if virus replication is suppressed by therapy (Rx, drugs or immunotherapy; ⊽ early after acute exposure, and virus replication is kept below threshold. †Death.

activation of the long terminal repeat (LTR) by other pathogens or cytokines [52], could upregulate replication-impaired viruses to exceed threshold. In contrast, an avirulent virus, which has lost the gene(s) required to induce disease, could replicate to any level and would not cause disease, regardless of host immunocompetence.

Viral threshold in murine systems

We hypothesize that host cellular immunocompetence is the major determinant for the viral threshold. To test this, the same RLV stock was titrated simultaneously in normal BALB/c mice and in their athymic nude counterparts. Indeed, the virus stock appeared to be 10 times more infectious in the T-cell-deficient mice than in normal animals (unpublished data). Subsequently, we rechallenged normal BALB/c mice, which had been exposed to low doses of pathogenic RLV but showed no viremia 3 weeks later, with 20 or 200 animal infectious doses (AID) of wild-type RLV. Overall, 17% of these mice resisted rechallenge with these doses which were fully infectious in naive control mice. Thus, exposure to subthreshold levels of fully pathogenic RLV induced protective immunity, which allowed some of the animals to resist rechallenge with higher doses of live virus. A fully pathogenic retrovirus given at low doses acted as a vaccine.

These data indicate that subthreshold exposure to a live, fully pathogenic virus can be controlled by the host and that protective antiviral immune responses can be induced, provided viral replication remains below threshold. Finally, although the pharmacologically attenuated live RLV vaccine protected many adult mice against high-dose challenge [46,48], the vaccination alone caused RLV disease when the animals were inadvertently coinfected with mouse hepatitis virus (unpublished data). This experiment reveals a serious hazard of replication-impaired live retrovirus vaccines: *success requires that host defenses outrace vaccine virus replication.* Because RLV was only attenuated by drugs interfering with its ability to replicate, the vaccine strategy failed when the animals were immunocompromised temporarily.

Evidence supporting the threshold hypothesis has also been provided by experiments in other murine systems. In SCID-hu mice transplanted with human fetal thymus, *nef*-deleted HIV-1 replicated poorly and caused no loss of human thymocytes [53] in contrast to chimeric mice

Table 1. Classification of attenuated retroviruses.

			Virus load exceeding threshold		AIDS	
Virus characteristics	Mechanism of attenuation	Examples	In normal host	In host with immune disturbance	In normal host	In host with immune disturbance
Replication-impaired	Interference with the 'mechanics' of replication	SIVΔ3	No	Yes	No	Yes
		SIVΔ*nef*	No	Yes*	No	Yes*
Avirulent	Loss of gene(s) for virulence		NA, virus load could be high	NA, virus load could be high	No	No
Pathogenic	None	SIV_{mac251} other SIV strains	Yes	Yes	Yes	Yes

Theoretically, retroviruses can be divided into three categories: replication-impaired, avirulent or pathogenic. While the first two groups of viruses are attenuated, they differ in the mechanism of attenuation. Replication-impaired viruses are attenuated in the mechanics of replication, while retaining the gene(s) encoding pathogenicity. Such a virus can become virulent if the relative loss of replicative capacity is restored by other factors. In contrast, avirulent viruses do not cause disease, even if the virus is fully replication competent. Consequently, no disease-defining threshold exists. NA, not applicable; *, expected outcome for SIVΔ*nef* in analogy to our data with SIVΔ3 [49].

inoculated with an equal amount of wild-type virus. However, when a 10 times larger dose of *nef*-deleted virus was given and the observation period extended, thymocyte depletion was seen in approximately 25% of the transplants (J. Zack, personal communication, 1995).

Viral threshold in simian systems

We found further evidence for a viral threshold effect from our SIV experiments in rhesus macaques. Initially, we attempted to study whether active and passive immunization could prevent infection of newborns after oral virus challenge. Epidemiological data indicate that 50–70% of vertically infected human infants acquire HIV-1 during birth [54]. The presence of serosanguinous secretions in neonatal gastric aspirates presents a significant risk for infection [55], implicating the neonatal alimentary tract as the portal of virus entry. We reasoned that oral vaccination with a live attenuated virus could induce protective immunity at the mucosal level.

Our experiments [49] were conducted with SIVΔ3 [56], an SIV mutant containing large deletions in the *nef* and *vpr* genes as well as in the negative regulatory element of the LTR. Experiments by other investigators had shown that *nef*-deleted SIV, although fully replication competent in T-cell lines, replicated only to a limited extent in adult macaques and caused no disease [57]. When persistently infected rhesus monkeys were challenged with pathogenic *nef*-positive SIV, they were protected [58]. Macaques vaccinated with *nef*-deleted SIV have resisted challenge with cell-free homologous [58,59] or heterologous virus [58] even at high doses [58]. Protection against challenge with cell-associated, wild-type virus has been achieved as well [59]. Curiously, however, protective responses developed only slowly. In a series of experiments with SIVΔ3, full protection against wild-type SIV challenge was seen only in a cohort of animals vaccinated 79 weeks before challenge but not in those challenged at 8 and 20 weeks [60]. Nevertheless, the success of *nef*-deleted SIV as an attenuated live virus vaccine has led to the proposal that similar strategies should be tested against human AIDS [58,61,62].

On the basis of the prior vaccine protection achieved, cell-free SIVΔ3 was given orally to 3 neonatal macaques [49]. Unlike vaccinated adults, who usually develop an initial peak of viremia but have very low to unmeasurable levels of infectious virus in PBL about 3 months post-inoculation, the orally exposed infants maintained persistently high virus loads and developed AIDS. The deletions in SIVΔ3 had not reverted. To test whether the SIVΔ3 had mutated elsewhere in the genome, we performed an *in vivo* transfer experiment. Blood was obtained from an infected macaque infant with AIDS; one-half was injected intravenously into an adult female, and the other half was administered orally to her infant at birth. The recipient mother had an initial peak of viremia and seroconverted, but from 8 weeks after virus exposure onward, no virus could be isolated from 10^6 PBL by cocultivation. In contrast, the infant had high virus loads and rapidly developed AIDS; at 34 weeks of age, it died with severely depleted CD4+ T cells and renal failure. Because both mother and infant were exposed to the same dose of virus (which was approximately 1.7 times the infectious dose used in previous experiments to vaccinate adult macaques [60]), viral factors can be eliminated as a cause for this difference in virulence. The route of virus exposure also does not explain SIVΔ3 virulence in neonates, based on recent experiments with intravenously infected infant macaques (unpublished data). Consequently, we believe that host factors are responsible for the differential pathogenicity of SIVΔ3 in the adult and neonate. According to the threshold hypothesis, SIVΔ3 replication remained below threshold in adult macaques, in contrast to infected infants, in which host factors were favorable for virus replication allowing the virus to exceed threshold. We classify SIVΔ3, and by inference SIVΔ*nef* [57], as replication-impaired rather than avirulent viruses (Table 1).

Because AIDS developed in several neonatally infected macaques, the deletion in *nef* and *vpr* are not sufficient to abrogate virulence of SIV. It follows that *nef* is not the major determinant for virulence in primate lentiviruses, based on the fact that SIVΔ3 induced AIDS in neonatal macaques. Rather, this gene product contributes to pathogenicity indirectly by increasing virus load, at least in adults. Various reports support this interpretation. In permanent T-cell lines, *nef*-deleted viruses and wild-type viruses replicate equally well, but in unstimulated primary cells, *nef*-positive viruses have a distinct although not absolute growth advantage [63,64]. Nef has also been shown to increase the infectivity of virions by accelerating the rate of proviral DNA synthesis [65]. Nef downregulates CD4 receptors [66,67]. The *nef* allele of SIV_{pbj14} has been shown to be a powerful cellular activator [68], allowing this virus to grow in unstimulated primary cells much better than other SIV variants [68,69]. Molecular analysis revealed a single amino-acid change in one of the functional domains of Nef [68]. When the residue at this position was mutated in the parental wild-type virus SIV_{mac239}, the resulting virus not only had the same *in vitro* growth characteristics as SIV_{pbj14}, but it also induced the acute disease characteristic of SIV_{pbj14} infection. Infected animals underwent massive intestinal lymphoproliferation [68]. In summary, these data support our interpretation that Nef upregulates virus replication by favoring virus production from unstimulated cells, leading to significant increases in virus load.

We postulate that because of the relative immaturity of the neonatal cellular immune system, which is associated with a functional deficit of antigen-presenting cells [70], SIVΔ3 replication could not be contained in neonatally infected macaques. In contrast, infected adults are able to

repress virus replication in PBL following the initial peak of viremia. In the race between virus replication and the generation of host cellular immune defenses, the virus won in neonatal animals, in contrast to adults. However, SIVΔ3 replication continues at a smoldering level even in adults, as shown by increasingly intense antibody responses [49]. Furthermore, *in situ* hybridization with lymph-node tissues from SIVΔ3-infected adult monkeys has revealed trapping of virus particles by follicular dendritic cells (unpublished data). We believe that SIVΔ3 replication can be contained by adult macaques as long as cellular immunity is intact and the animal is not coinfected with pathogens known to stimulate the SIV LTR directly or indirectly. Interestingly, replication of *nef*-deleted SIV seems to have been reactivated in an adult rhesus monkey that developed a lymphoproliferative disease 29 months post-inoculation [71]. This animal harbored a significantly higher proviral burden compared with healthy adult monkeys infected with *nef*-deleted SIV mutants [71].

Recent experiments conducted during titration of SIV by intra-rectal exposure in adult rhesus monkeys lends additional support for the threshold hypothesis. Pauza and coworkers [72–74] exposed animals to various doses of pathogenic virus, ranging from 0.1 to 1000 AID (as measured by intravenous inoculation). Animals inoculated intra-rectally with high doses of SIV seroconverted, were positive by PCR and cocultivation of PBL, and exhibited rashes and lymphadenopathy. In contrast, animals inoculated intra-rectally with low doses (0.1–10 AID) failed to seroconvert, remained negative by PCR and viral culture, and remained asymptomatic. Thus, no evidence of a systemic infection was seen. When these monkeys were rechallenged intra-rectally with 200 AID of SIV, their serological, virological and clinical status remained unchanged, whereas the naive control animals became viremic and developed disease. These results indicate that exposure to a low-dose inoculum resulted in resistance to superinfection with a homologous virus challenge.

The vaccine protection provided by low-dose pathogenic SIV is not dependent on the initial route of virus exposure, as macaques inoculated intravenously with subinfectious doses of SIV also resisted subsequent intra-rectal challenge with high-dose SIV that led to infection in all naive controls [75]. Subsequent studies have implicated T-cell immunity in this protective response [72,74,75]. Apparently, the low-dose inocula allowed the hosts to develop cellular immune defenses, which limited the initial infection and were protective even against higher challenge doses. Thus, subthreshold exposure to the fully pathogenic SIV induced a vaccine effect, analogous to our findings in the RLV system.

The threshold effect in HIV-1-exposed humans

Suggestive evidence supporting the threshold hypothesis is accumulating from studies of various human cohorts exposed to HIV-1 [76–87]. Following needlestick injuries, which typically involve small doses of infectious virus [88], health-care workers have developed specific anti-HIV-1 cellular immune responses without signs of infection, such as seroconversion, positive virus cultures or PCR positivity [76]. High-risk individuals, commercial sex workers, sexual partners of infected individuals, and children born to infected women have also exhibited the same pattern of immune responsiveness without documented infection [77–87]. Conceivably, these individuals were exposed naturally to low virus inocula, which induced protective immune responses, analogous to the animal experiments described above.

Even more intriguing are reports that suggest transient HIV-1 infection and clearance in children born to infected mothers [89–97]. In the most carefully studied case [92], a child had no signs of active HIV-1 infection at age 6 years, although during the first few months of life he was intermittently positive by virus isolation and PCR. In an attempt to exclude laboratory artifacts, partial viral sequence analyses were performed, which revealed close homology between strains isolated from mother and child. Although some scientists remain skeptical, these findings can be consistent with the threshold hypothesis: infection with low doses of virus can induce protective cellular immune responses capable of eliminating infected cells, resulting in HIV-1 clearance.

Although most HIV-1-seropositive individuals eventually become immunosuppressed, a small fraction of seropositive persons do not show signs of disease progression, even after more than 10 years [98–100]. Typically, viral loads in these long-term non-progressors remain low. It is possible that some of these individuals harbor viruses that are either replication impaired or truly avirulent. In a few long-term non-progressors studied thus far, deletions in the *nef* gene have been detected [101,102]. However, host factors rather than viral determinants could dictate the outcome equally well, as shown in our RLV and neonatal SIV experiments. We postulate that in long-term non-progressors the critical balance between rate of virus replication and host immune responsiveness was tipped in favor of the host. In the absence of an animal model for HIV-1 disease, no definitive conclusions can be drawn regarding the virulence of virus strains in long-term non-progressors.

The threshold effect in non-retroviral systems

The balance between host immunity and virus replication has been examined in detail in mice infected with lymphocytic choriomeningitis virus (LCMV) [103,104].

In this system, specific antiviral cellular immune responses *per se* can cause severe disease, depending on viral factors as well as host genetic determinants [105,106]. T-cell-mediated immunopathology is an important determinant of the clinical outcome following LCMV infection. Whether similar mechanisms contribute to the differential virulence of SIVΔ3 in neonatal versus adult macaques remains to be determined.

Threshold effects have been observed also with other intracellular microbial pathogens, for example in a study of BALB/c mice inoculated with a small number of infectious *Leishmania* parasites [107]. Animals exposed to small inocula acquired resistance to a large challenge dose, which normally induces progressive disease in this strain of mice. Adoptive transfer of splenocytes from mice exposed to low doses conferred protection to naive recipients, and the cellular immune responses were shown to be T-helper-1-like [107]. As in the RLV and SIV experiments described previously, subthreshold infection with wild-type pathogens induced protective cellular host immune responses.

Summary

By considering the dynamic relationship between retroviruses and their hosts, we have developed a unifying hypothesis to explain such disparate clinical phenomena as differential pathogenicity of a given virus in adults and neonates, transient infection with clearance of provirus-containing cells, long-term non-progression and vaccine effects of fully pathogenic viruses. The threshold hypothesis predicts that an opportunity exists during acute retroviral infection to influence the ultimate clinical outcome: if virus replication is kept below threshold by any means, including drug therapy or passive immunoprophylaxis with neutralizing antibodies, the host will prevail and win the race.

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Correlates of Immune Protection after Vaccination with Attenuated Live Murine Leukemia Virus

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THE FIRST SUCCESSFUL RETROVIRAL VACCINATION was carried out in 1959 by Friend.[1] Since then, numerous experiments have been conducted on murine leukemia virus vaccines, with varying success. The approaches have included killed virus,[2] subunit vaccines,[3] recombinant vaccinia viruses expressing viral gene products,[4,5] peptide vaccines,[6] and live attenuated retroviruses. Methods to attenuate pathogenic retroviruses have included prolonged passage through tissue culture,[7] use of viral strains that cannot replicate in certain host animals due to genetic restriction,[5] and use of live pathogenic virus blocked pharmacologically from replicating.[8] It would be of great interest to compare the relative potency of various vaccine strategies directly. To be rigorous, such a comparison would require genetically identical host animals, a well-defined vaccine preparation, and a standardized challenge dose of virus. While no comparative analysis involving all possible vaccine strategies has been published, the relative effectiveness of certain vaccines against Friend virus infection and disease has been compared.[4,5]

Friend virus is a complex consisting of at least two components: the replication-competent helper Friend murine leukemia virus and a replication-defective spleen focus-forming virus defective in *env*.[9] Friend virus causes severe immunosuppression in some inbred mice, which develop neutralizing and cytotoxic antibodies but are incapable of eliminating infected cells. Their immune T cell function is suppressed. Friend virus replication is controlled by a locus in the host genome termed *Fv-1*, which has two alleles ($Fv\text{-}1^{nn}$ and $Fv\text{-}1^{bb}$). On the basis of host factors determined by this locus, Friend virus can be separated into Friend virus-N (N-tropic) and Friend virus-B (B-tropic) strains. Friend virus-N virus replicates to high titers and causes disease only in mice of the $Fv\text{-}1^{nn}$ genotype. The converse holds for Friend virus B. Only low-level infection and no disease is seen when Friend virus-N virus is injected into mice of the $Fv\text{-}1^{bb}$ genotype and vice versa.

This system was exploited as a live attenuated virus vaccine strategy and compared with other vaccination approaches[4,5] (Table 1). Two closely related strains of mice of the $Fv\text{-}1^{bb}$ genotype were examined. The success of whole killed virus and live recombinant vaccinia virus expressing the Friend murine leukemia virus *env* gene product depended critically on the *H-2* haplotypes of the vaccinated mice; little success was achieved in *H-2(a/a)* mice with either vaccine strategy. Only live virus, attenuated by host genetic restrictions, protected both *H-2(a/a)* and *H-2(a/b)* mice. The strongest cytotoxic T lymphocyte responses in vaccinated mice were elicited by live attenuated virus, but the neutralizing antibody titers were not well correlated with protection. Clearly, in this genetically defined system, the attenuated live virus vaccine approach was superior to the other strategies tested. Most importantly, the potency of live attenuated Friend virus N as a vaccine was not dependent on host haplotype. The potency of live attenuated virus was also evident in our vaccine experiments.[8]

We showed that live, attenuated Rauscher murine leukemia virus (RLV) induces potent immune protection against challenge with a high dose of live virus (approximately 5000 AID_{50} [50% animal infectious doses]) (Ref. 8, and our unpublished data). These vaccine studies evolved from our prior work with postexposure drug prophylaxis, in which a short course of AZT (zidovudine) plus interferon α prevented viremia and disease in all RLV-exposed mice.[10] Most of these mice resisted rechallenge with live virus in the absence of further therapy. By definition, live, pathogenic RLV acted as a pharmacologically attenuated viral vaccine.

To determine the correlates of immune protection, passive transfer experiments were performed, which revealed:

- Passive immune serotherapy provided only partial protection[8]
- No neutralizing antibodies were present[8]
- Retroviral immunity could be conferred to naive recipients by immune T cells alone[8]
- Both $CD4^+$ and $CD8^+$ immune T cells were required for full protection[11]
- Immunity was long lasting (our unpublished data)

This series of experiments lends strong support to the threshold hypothesis that we published (Refs. 12–14; Ruprecht *et al.*, "Attenuated" Simian Immunodeficiency Virus in Macaque

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TABLE 1. COMPARATIVE EFFECTIVENESS OF FRIEND VIRUS VACCINES AGAINST CHALLENGE WITH FRIEND VIRUS-B[a]

	Leukemic mice (%)		*Friend virus-specific immunity in H-2(a/b) mice*		
Vaccine	*H-2(a/b)*	*H-2(a/a)*	*Neutralizing antibody (% positive)*	*CTL response*	*T cell proliferation*
None	96	95	0	−	−
Whole killed Fr-MLV (formalin fixed + CFA)	0	56			
Vaccinia-Fr-MLV *env*	5	77	16	+	+
Vaccinia–influenza HA	95	95	0	−	−
Live attenuated	2	0	58	4+	+

[a]Adapted from Earl *et al.*[4] and Morrison *et al.*[5]

Abbreviations: CTL, cytotoxic T lymphocyte; Fr-MLV, Friend murine leukemia virus; CFA, complete Freund's adjuvant; vaccinia-influenza HA, recombinant vaccinia virus expressing influenza virus hemagglutinin A.

Neonates, this issue), according to which retroviral pathogenicity can become apparent only if the level of viral replication exceeds a predetermined threshold in a given host. Subthreshold replication, however, will not lead to disease, even if the virus is fully pathogenic.

We postulate that the postexposure drug therapy reduced the replication of pathogenic RLV below threshold (see Refs. 12–14; Ruprecht *et al.*, "Attenuated" Simian Immunodeficiency Virus in Macaque Neonates, this issue) and allowed the mice to generate protective cellular immune responses. To test this idea, RLV-exposed mice were sacrificed serially during drug coverage. DNA polymerase chain reaction analysis showed that the proviral signals increased in both untreated and treated RLV-exposed mice early after infection, but drug-treated mice gradually lost proviral sequences. Most immune mice had no RLV DNA. Immunosuppression of immune mice with cyclosporin A did not reactivate RLV, but cyclosporin A treatment after acute infection prevented clearance of RLV by AZT plus interferon α. Likewise, drug therapy alone did not allow nude mice to clear RLV, in contrast to immunocompetent mice.[13] To test the threshold hypothesis further, normal mice were exposed to low doses of live, pathogenic RLV, which were infectious in nude mice. Normal mice with no sign of infection were rechallenged with higher doses of live virus; 15% were immune.

While the pharmacologically attenuated live RLV vaccine strategy provided protection to hundreds of adult mice after a high-dose challenge, a significant problem became obvious in the use of replication-impaired retroviral vaccines. When adult mice were inadvertently coinfected with mouse hepatitis virus, they became persistently infected with RLV during the vaccination phase and developed disease without live virus rechallenge. Because the virus in the vaccine was attenuated pharmacologically only by interference with its ability to replicate (attenuated in the mechanics of replication), the vaccine strategy failed when the animals were temporarily immunocompromised. This result signals a serious danger of vaccine strategies involving replication-impaired live retroviruses: success is based on host defences outracing vaccine virus replication. In immunocompromised hosts, however, the vaccine virus could replicate above threshold and reveal its underlying virulence.

In conclusion, we have shown that retroviral immunity is correlated with cellular immune responses; protection against a high dose of virus challenge can be achieved in the absence of measurable neutralizing antibodies. While the live "attenuated" viral vaccine approach has inherent dangers, it provides important insight into the nature of protective immunity. We conclude that the development of vaccines against retroviral infections should emphasize induction of strong cellular immune responses, involving both $CD4^+$ and $CD8^+$ immune T cells. If the presence of neutralizing antibodies is used as the major criterion for evaluating the effectiveness of candidate vaccines, vaccination strategies resulting mainly in protective cellular immunity may be missed.

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Live attenuated HIV as a vaccine for AIDS: pros and cons

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Anti-HIV-1 vaccines must be safe and effective. In macaques, live attenuated simian immunodeficiency viruses have provided the best protection to date. Similar results were obtained earlier in murine leukemia virus systems in which protection correlated with cellular immunity but not with neutralizing antibodies. Attenuated primate lentiviruses tested thus far have been replication-impaired but may still harbor genetic determinants encoding virulence. Other safety issues concern insertional oncogenesis, genetic instability, vertical transmission and differential pathogenicity in adults and newborns, and viral persistence with possible reactivation during intercurrent illness. Long term safety studies are needed to assess the risks associated with live attenuated retrovirus vaccines.

Key words: attenuated live retrovirus vaccines / avirulent viruses / correlates of retroviral immunity / replication-impaired viruses / threshold hypothesis

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Problems for HIV-1 vaccine development

Given the continued escalation of the HIV-1 epidemic worldwide, no one disputes the need for a safe and effective vaccine. The majority of HIV-1 infections are acquired by mucosal contact. Consequently, vaccines need to protect against virus spread following mucosal exposure. Currently, it is not known whether a successful anti-HIV-1 vaccine needs to induce sterilizing immunity.

In comparison to other viral infections, vaccine development against HIV-1 faces significant obstacles. The virus is prone to mutate because its replication depends on reverse transcriptase which lacks a proof-reading function. The special mechanisms of reverse transcription also lead to frequent recombinations.[1,2] The plasticity of the HIV-1 genome makes the induction of broadly protective immune responses mandatory. Virus strains isolated from various parts of the world show significant divergence; thus far, subgroups O and M, which contains eight different clades, have been identified.[3] Recombination between different clades has been found,[3] which not only demonstrates the ability of different HIV-1 subtypes to superinfect and recombine, but also implies that immune responses generated in an infected person against virus of one clade do not protect against viruses of different clades.

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Live attenuated viruses as vaccines against viral diseases

Live attenuated virus vaccines have been developed against members of different virus families and protect against various human viral diseases including poliomyelitis, mumps, measles, rubella, yellow fever, and most recently, chickenpox (for review, see ref 4). The most striking success, the world wide eradication of smallpox, was due to the use of live attenuated vaccinia virus. The immunity induced by live attenuated viruses is typically long-lasting, broad-spectrum, and can confer protection against mucosal infection routes.

Mechanisms for viral attenuation

Currently licensed live virus vaccines have been attenuated by various approaches (for review, see ref 4). Attenuated Sabin polio viruses types 1 and 3 were generated by repeated passage through cells of unnatural hosts.[5] These strains accumulated several mutations which conferred temperature sensitivity and loss of neurovirulence after direct injection at high doses into monkey spinal cords. The Sabin type 2 strain is a naturally occurring human variant lacking neurovirulence.[5] Prolonged serial passage through

cultured human cells can also lead to attenuation, as shown for the Oka strain of varicella virus. To vaccinate against smallpox, Jenner used a live animal virus to protect against a pathogenic human virus with antigenic cross-reactivity to the animal virus. If animal viruses are poorly adapted to human hosts, replication is restricted but sufficient to generate protective immunity against a pathogenic human virus. Artificial restriction of virus replication to prevent target organ infection is used to vaccinate against adenovirus which causes epidemic respiratory tract illness. Live, wild-type virus is administered orally in enteric coated tablets. The virus replicates locally in the intestinal tract which induces protective immunity against subsequent natural respiratory tract infection.

Defining viral attenuation

We have proposed to classify live attenuated viruses into two broad categories according to the mechanism of attenuation[6-8] (Table 1). The first category encompasses viruses attenuated in their ability to replicate, whereas the second category includes viruses attenuated in their ability to cause disease regardless of their replicative capacity. An example for a replication-impaired, live attenuated virus is orally administered adenovirus given in enteric coated tablets. In contrast, live attenuated Sabin polio virus strains are predominantly attenuated in their neurovirulence while maintaining their ability to replicate in the gastro-intestinal tract. Attenuated viruses may exhibit a mixed pattern within a wide spectrum of partial replication impairment as well as partial loss of pathogenicity.

The role of animal models in the development of live attenuated virus vaccines

Animal models play an important role in testing the safety and efficacy of attenuated viruses. Virus challenges can be performed with defined inocula via defined routes of virus exposure. Determination of the correlates of immune protection will provide important guidelines for evaluating vaccine effects in subsequent human trials.

Animal models can be divided generally into models for viremia or models for disease. Virulence can be examined solely in the latter, since the former only permit studying the replicative capacity of a virus. The availability of primate models for neurovirulence played a key role in developing the Sabin polio vaccines.[5] AIDS vaccine research is hampered by the lack of an animal model for HIV-1 disease. Chimpanzees can be infected, but after more than 10 years of follow-up, only one out of over 200 animals has developed AIDS.[9] Realistically, attenuated HIV-1 mutants can only be assessed for impairment of replicative power but not for attenuation in virulence. If only humans are susceptible to HIV-1 disease, residual pathogenicity of replication-impaired mutants may not be predictable during pre-clinical evaluation of candidate attenuated HIV-1 vaccines. While attenuated live varicella vaccines have been developed without the aid of an animal model for virulence,[4] the morbidity and mortality of varicella cannot be compared to that of HIV-1. Residual virulence or reversion to virulence in live attenuated varicella would lead to a relatively harmless exanthematous, transient illness, whereas the risks in HIV-1 are considerably higher.

The lack of an animal model for HIV-1 disease has led to the use of surrogate animal/lentivirus models.

Table 1. Viral attenuation and potential for causing diseases

		Above threshold replication		Pathogenicity	
Mechanism of attenuation	Virus nomenclature	in normal host	in co-infected or immunocompromized host	in normal host	in co-infected or immunocompromized host
Decreased ability to replicate	Replication impaired	No	Yes	No	Yes
Loss of pathogenicity	Avirulent	(Any level)	(Any level)	No	No
None	Pathogenic	Yes	Yes	Yes	Yes

Mechanisms of retroviral attenuation. Theoretically, a retrovirus can be attenuated for its ability to replicate while retaining the gene(s) encoding virulence. Such a virus could become dangerous if the relative loss of replicative power were compensated by other factors. An avirulent virus, on the other hand, would not cause disease, regardless of virus load. Consequently, no disease-defining threshold exists for this avirulent virus in the host (parentheses). Printed with permission from Ruprecht *et al.* AIDS Res Hum Retroviruses 1996; 2: 459-460.

For HIV-1 vaccine development, simian immunodeficiency virus (SIV)[10] infection of rhesus monkeys is considered widely as the most relevant model system.[11,12] HIV-1 and SIV not only have extensive homologies in genome structure and nucleic acid sequence, but the patterns of viremia and disease are similar in man and monkey.[13] Both viruses infect similar target cells, and key features of human AIDS are also seen in SIV-infected rhesus monkeys which show high levels of virus replication, $CD4^+$ T-cell depletion, immunosuppression and central nervous system disease.[13]

Live attenuated murine leukemia viruses: relative vaccine potency and correlates of immune protection

Retroviral infections in different species share many biological features, even though clinical manifestations vary greatly. The need to integrate and establish permanent infection is common to all retroviruses, defining them as genetic parasites. Spread via cell-free and cell-associated virions occurs in all systems, and host defenses need to be mobilized against both routes of attack. We postulate that immunoprophylaxis against retroviral infections in different species will be achieved through common mechanisms. Thus, information gained from successful vaccine strategies against other retroviruses should be considered in developing anti-HIV-1 vaccines. Murine leukemia virus (MuLV) vaccine studies, due to the unique advantage of using small inbred animals, have yielded data on two key aspects of vaccine development: the relative potency of different vaccines and the correlates of immune protection. A brief summary emphasizing the role of attenuated live viruses as vaccines is given here.

The first successful retroviral vaccination in 1959[14] was followed by numerous MuLV vaccine experiments using killed virus,[15] subunit vaccines,[16] oligopeptides,[17] recombinant vaccinia viruses expressing viral gene products,[18,19] and live attenuated viruses,[19,20] used as early as 1962.[20] MuLV was attenuated either on the basis of host age[20] or lack of genetic susceptibility to MuLV infection of the host,[19] by prolonged passage through tissue culture,[21] or by exogenous pharmacological inhibition of viral replication[22,23] (Table 2). The relative potency of some anti-Friend virus (FV) vaccines has been studied[18,19] (Table 3).

FV, a virus complex consisting of replication-competent helper virus (Friend MuLV) and replication-defective spleen focus-forming virus (SFFV) (for review, see ref 24), causes severe immune T-cell dysfunction in some inbred mice which develop neutralizing antibodies but cannot eliminate infected cells. FV replication is controlled by a locus in the mouse genome, *Fv-1*. Based on its two alleles, $Fv\text{-}1^{nn}$ and $Fv\text{-}1^{bb}$, FV is separated into two strains, FV-N and FV-B. Virus replication is restricted and no disease develops when FV-N is injected into mice of the $Fv\text{-}1^{bb}$ genotype and *vice versa*. This system was exploited as a live attenuated virus vaccine strategy and compared directly to other approaches in two closely related mouse strains of the $Fv\text{-}1^{bb}$ genotype[19] (Table 3). The success of whole killed virus and live recombinant vaccinia virus expressing Fr-MuLV Env depended critically upon the host major histocompatibility genes; neither vaccine strategy protected H-2(a/a) mice. Only live 'genetically attenuated' FV-N induced

Table 2. Live attenuated MuLV vaccines

Virus	Vaccine	Challenge	Results	Immunological studies	Refs
Mo-MuLV	Live virus in adult mice (non-pathogenic)	$MoMuLV^+$ leukemic cells	Protection from transplanted leukemia		20
RLV	Live virus, attenuated by tissue culture passage	$MoMuLV^+$ leukemic cells	80% protection from transplanted leukemia		21
FV	Live, attenuated FV-N	FV-B	Viremia suppressed, no disease	Strong antiviral CTL; poor correlation with neutralizing antibody	19
RLV	Live, pathogenic virus, blocked by drugs	RLV (pathogenic); no drugs	94–100% protection against viremia	Adoptive transfer: cellular immunity correlates with immune protection. No neutralizing antibodies were detected.	23

Summary of live-attenuated MuLV vaccine experiments. MoMuLV, Moloney murine leukemia virus; RLV, Rauscher murine leukemia virus; FV, Friend murine leukemia virus complex.

Table 3. Comparative effectiveness of FV vaccines against FV-B challenge

Vaccine	% Leukemic mice		FV-Specific immunity in H-2(a/b) mice		
	H-2(a/b)	H-2(a/a)	Neutralizing antibody % positive	CTL	T-Cell proliferation
None	96	95	0	–	–
Whole killed Fr-MuLV (formalin-fixed + CFA)	0	56			
Vaccinia-Fr-MuLV*env*	5	77	16	+	+
Vaccinia-influenza HA	95	95	0	–	–
Live attenuated (FV-N)	2	0	58	4+	+

CFA, complete Freund's adjuvant; vaccinia-influenza HA, recombinant vaccinia virus expressing influenza virus hemagglutinin A. After Earl *et al*[18] and Morrison *et al*.[19]

broad protection and did not depend on host haplotypes. The potency of live attenuated virus was also evident in our Rauscher MuLV (RLV) vaccine experiments.[23,25]

The correlates of immune protection after live attenuated MuLV vaccination

In the comparative FV vaccine series[18,19] (Table 3), neutralizing antibody titers did not correlate with protection; even though most mice vaccinated with FV-N were protected, neutralizing antibodies were detected only in 58% of vaccinees. In contrast, the strongest cytotoxic T lymphocyte (CTL) responses among all vaccinees were elicited by FV-N.

We determined the correlates of retroviral immunity directly by adoptive transfer.[23,25] We found that live, drug-attenuated RLV induced potent immune protection against challenge with high-dose live virus (approximately 5,000 animal infectious $doses_{50}$) (ref 23, and unpublished data). These vaccine studies evolved from our prior work with post-exposure drug prophylaxis, in which a short course of therapy prevented viremia and disease in all RLV-exposed mice.[22] Most of these mice resisted live-virus rechallenge in the absence of further drug therapy; by definition, they were immune. Using this vaccine approach, adoptive transfer experiments were performed which revealed:[23,25] (1) passive immune serotherapy only provided partial protection; (2) no neutralizing antibodies were present; (3) retroviral immunity could be conferred to naive recipients by immune T cells alone; (4) both $CD4^+$ and $CD8^+$ immune T cells were required for full protection; and (5) immunity was long lasting.

Retroviral immunity correlated with cellular immune responses; protection against high-dose virus challenge was achieved in the absence of measurable neutralizing antibodies. We conclude that vaccine development against retroviral infections should emphasize induction of strong cellular immune responses, *involving both $CD4^+$ and $CD8^+$ immune T cells.* Evaluation of candidate vaccines using neutralizing antibodies as a major criterion for effectiveness may miss vaccination strategies resulting mainly in protective cellular immunity.

Data from different MuLV studies have demonstrated that powerful protection against pathogenic virus is provided by live attenuated vaccines. While the vaccine strategies described were effective, they would not be considered safe and applicable for a genetically heterogeneous human population. Furthermore, a serious problem involving the use of replication-impaired retrovirus vaccines became obvious in our vaccine series involving drug-attenuated, live RLV. This vaccine strategy provided protection to hundreds of adult mice against high-dose challenge, but when adult mice were inadvertently co-infected with mouse hepatitis virus, the mice became persistently infected with RLV during the vaccination phase and developed disease, without live virus re-challenge. Because the vaccine virus was only attenuated pharmacologically by interference with its ability to replicate (attenuated in the mechanics of replication), the vaccine strategy failed when the animals were temporarily immunocompromised (unpublished).

Live attenuated SIV

The first attenuated SIV was isolated after prolonged passage of SIV_{mac251} in human cell lines.[26] This virus, termed 1A11, differs at several restriction sites when compared to wild-type, cloned SIV_{mac239} and contains an open reading frame for *nef*. While 1A11 replicates well in cultured cells, replication in infected animals is restricted. Macaques persistently infected with 1A11

were only protected from early disease but not infection following challenge with wild-type virus.[26] To elucidate the molecular determinants of attenuation, recombinants between the parental virus SIV_{mac239} and 1A11 were generated and tested as vaccines. An important correlation between the ability of an attenuated virus to replicate in animals and its ability to generate protective responses against challenge with pathogenic SIV emerged: the level of protection increased in parallel to the ability of the virus to replicate in the animal.[27]

An alternate strategy for developing attenuated SIV vaccines was based on the observation that the replication of a molecular SIV construct deleted in the auxiliary *nef* gene was restricted in adult macaques following intravenous injection.[28] Although these animals were persistently infected with low levels of virus, they maintained normal $CD4^+$ T-cell counts and developed no signs of immunodeficiency during more than 3 years of follow-up. When challenged with pathogenic SIV_{mac251} containing an open *nef* reading frame, these animals were protected from infection with wild-type SIV and development of AIDS.[29] Almond *et al*[30] also showed that an SIV mutant with an in-frame deletion of 12 amino acids in Nef induced protective responses in monkeys against challenge with cell-free and cell-associated wild-type virus.

These data led to the hypothesis that *nef* is a major determinant of virus load and pathogenicity *in vivo* for immunosuppressive primate lentiviruses.[28] The *nef* gene product, a myristylated phosphoprotein of 34 kD apparent molecular weight, down regulates CD4 surface antigen expression[31-34] but is not required for virus replication in cultured T-cell lines. Other Nef functions are more controversial; various effects on transcription from the HIV-1 long terminal repeat (LTR) have been described (for review, see ref 34). According to more recent reports,[35,36] Nef facilitates virus replication in unstimulated peripheral blood mononuclear cells and enhances viral infectivity by stimulating proviral DNA synthesis.[37]

Based on the successful use of *nef*-deleted mutants as live attenuated virus vaccines in macaques, analogous HIV-1 mutants have been proposed as vaccines to protect humans against AIDS.[29] However, *nef*-deleted HIV-1 viral constructs have failed to protect chimpanzees.[38]

'Attenuated' SIV in macaque neonates

Recently, we showed that SIVΔ3, a mutant deleted not only in *nef* but also in *vpr* and in the negative regulatory element (NRE) of the LTR,[39] induced lethal AIDS in half of the orally infected macaque neonates and signs of disease in the surviving infants;[6] SIVΔ3 remained attenuated in adult animals after intravenous infection and induced protective responses against challenge with wild-type SIV.[40] Infected infants had persistently high virus loads, in contrast to infected adults.[6,40] *Nef* and *vpr* remained deleted in the diseased neonates. To test whether other regions of the SIV genome had undergone mutations affecting virulence, we injected SIVΔ3-infected blood from a macaque infant with AIDS intravenously into an adult macaque. The recipient adult seroconverted and had positive virus cultures for 10 weeks post-inoculation, but virus replication in the peripheral blood was suppressed subsequently. In contrast, a neonatal macaque given the same volume of SIVΔ3-infected blood orally had persistently high virus loads and died of AIDS at 34 weeks of age. This transfer experiment confirmed that SIVΔ3 remained virulent in the neonate and attenuated in the adult macaque.

We propose to classify SIVΔ3, which lacks a functional *nef* gene, as a replication-impaired vaccine virus, according to the definitions introduced earlier (Table 1). Since this virus induced lethal AIDS in macaque infants, the *nef* gene is not the major molecular determinant for virulence; rather, this gene influences virulence only indirectly by modulating virus load. Consequently, SIVΔ3 cannot be considered to be an avirulent virus. The latter would not cause disease despite high levels of replication, not even in immunocompromised hosts. In contrast, replication-impaired viruses are considered to be attenuated only in the mechanics of replication.

The threshold hypothesis

The differential pattern of SIVΔ3 viremia and pathogenicity in adult and infant macaques can be explained by the threshold hypothesis,[6-8] according to which retroviral virulence can only became apparent after the virus load exceeds a pre-determined threshold in a given host. If virus replication is repressed by any mechanism(s) and remains below threshold, disease will not ensue, even if the virus contains the intact gene(s) required for virulence (Figure 1). We postulate that in mucosally infected macaque neonates, replication of SIVΔ3 was unrestricted and exceeded the threshold, whereas host

factors (as opposed to loss of virulence) limited replication in intravenously infected adults. Suppression by host immune responses in adults but not neonates may account for the age-related differential pathogenicity of SIVΔ3.

Our RLV data provide direct evidence for the threshold effect. Protective immune responses were generated when fully pathogenic virus was used as 'vaccine'; replication was suppressed with antiviral drugs. We postulate that drug therapy reduced the replication of pathogenic RLV below threshold and allowed the mice to generate protective cellular immune responses. To test directly for low levels of replication during the initial phase of drug coverage, RLV-exposed mice were sacrificed serially. By DNA PCR analysis, proviral signals increased in both untreated and treated, RLV-exposed mice early after inoculation, but drug-treated mice gradually lost proviral sequences. Most immune mice had no RLV DNA (unpublished data). In contrast to immunocompetent mice, drug therapy alone did not allow T-cell deficient, nude mice to clear the same dose of RLV.[41] To further test the threshold hypothesis, normal mice were given low doses of pathogenic RLV, which were infectious in nude mice. No drug therapy was given. Normal mice without signs of infection 20 days later were rechallenged with higher doses of live RLV; 15% were immune (unpublished). These animals had not only cleared the initial infection, but also generated immunity against challenge with higher virus doses.

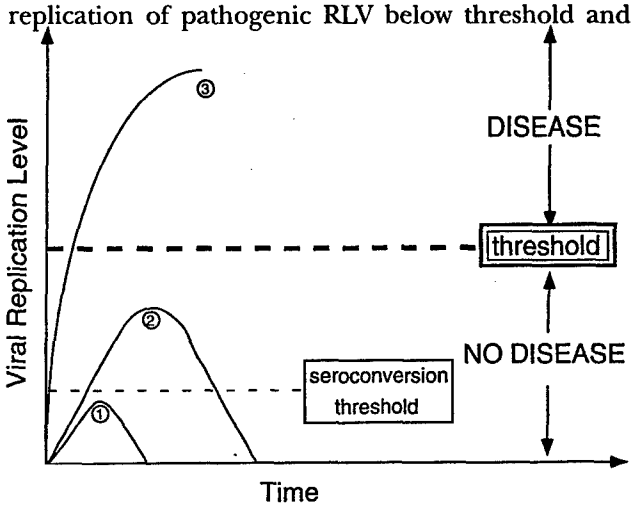

Figure 1. Host-retrovirus interactions. Development of disease after infection with a retrovirus occurs only after the level of virus replication has exceeded the threshold. Even a fully pathogenic virus can exhibit a pattern of transient viremia, abortive infection or latency if the level of replication does not reach the threshold. Host defenses, antiviral drug- or immunotherapy could be used to restrict virus replication. (1), transient viremia, abortive or latent infection, no seroconversion; (2), transient viremia, abortive of latent infection, with seroconversion; (3), persistent viremia, with seroconversion and disease development because virus replication exceeds the threshold. Printed with permission from Ruprecht *et al*, AIDS Res Hum Retroviruses 1996; 12: 459-460.

Table 4. Potential dangers of live attenuated HIV-1 vaccines

- residual virulence in a virus attenuated only for its ability to replicate
- virus persistence, with possible reactivation and high levels of replication during intercurrent illness
- generation of virulent vaccine virus mutants due to infidelity of reverse transcriptase and high recombination rates
- insertional mutagenesis resulting in neoplasia
- vertical transmission and increased pathogenicity in newborns
- slow development of protective responses; false sense of security during period of inadequate vaccine protection

Potential efficacy of a 'live attenuated' HIV-1 vaccine?

Recently, a cohort of female commercial sex workers in West Africa infected with HIV-2 was reported to have a 68% decrease in the rate of infection with HIV-1 as compared to uninfected women, even though the incidence of other sexually transmitted disease was actually higher in the HIV-2-positive group.[42] The virulence of HIV-2 was studied in a prospective study of registered Senegalese commercial sex workers.[43] Among women who seroconverted during the 7-year observation period, AIDS-free survival was 100% among HIV-2-infected women but only 67% among those who became HIV-1 positive. Loss of $CD4^+$ cells was also significantly lower in HIV-2 seroconverters than in women who became HIV-1 seropositive. While these results establish the lower virulence of HIV-2 as compared to HIV-1, the former virus has been isolated from AIDS patients without evidence of HIV-1 infection.

Did HIV-2 act as a live 'partially attenuated' vaccine to prevent infection with HIV-1? While no mechanisms were reported to explain the 68% risk reduction,[42] the intriguing possibility exists that host immune responses induced by HIV-2 are cross-protective against infection with the more virulent HIV-1. Alternatively, viral interference mechanisms could account for the lowered risk of HIV-2-infected women to acquire HIV-1 infection. Discovering the

cause for this apparent cross protection may yield important clues for anti-HIV-1 vaccine development. It should be stressed, however, that no one is currently advocating the use of HIV-2 as a candidate live 'attenuated' vaccine against HIV-1.

Potential dangers of live attenuated HIV-1 vaccines

Major potential dangers of live attenuated HIV-1 vaccines are summarized in Table 4.[7] Given the current lack of a practical animal model for HIV-1 disease, residual virulence of a vaccine virus weakened primarily in its ability to replicate cannot be predicted in preclinical studies. If host defenses are dampened, the virus replication threshold could be exceeded, allowing AIDS to develop. This may have occurred in our experiments with SIVΔ3-infected neonatal macaques and in mice given live, pathogenic RLV which were replication-suppressed by antiviral drugs. This 'attenuated' RLV vaccine strategy, while generating strong protective cellular immune responses in many normal adult mice,[23,25] failed in mice inadvertently co-infected with mouse hepatitis virus; these mice developed RLV disease without challenge (unpublished).

The threshold could also be surpassed if expression of a replication-impaired vaccine virus is upregulated and the relative loss of replicative capacity is compensated by other mechanisms.[6-8] Human vaccine recipients will likely harbor other pathogens which could induce certain host cytokines known to stimulate HIV-1 transcription. Alternatively, gene products encoded by other pathogens could transactivate the LTR, which again could allow virus replication to surpass the threshold. In contrast to other attenuated viruses, attenuated retroviruses can persist either as latent integrated proviruses or as slowly but actively replicating viruses. Other factors could upregulate vaccine virus replication in addition to co-infecting pathogens, such as exposure to ultraviolet radiation,[44] therapy with certain medications, or even aging. If the vaccine virus has residual virulence, the threshold could be surpassed and disease could develop, even after a long time interval following vaccination.

Continued low level virus replication in healthy vaccinees and, to a greater extent, stimulation of virus replication during intercurrent illness could carry another danger: generation of mutant viruses. Attenuated HIV-1 replication depends on reverse transcriptase with its well recognized infidelity due to the lack of proof reading function and tendency for recombination.[1,2] Animal retroviruses are known for their ability to scavenge cellular genes and incorporate them into the viral genome as oncogenes.[24] Consequently, each round of replication carries a finite risk of generating unfavorable mutations. In this context, it is important to mention that reversion to virulence was observed for SIV mutants containing truncated gp41[45] and deletions in *nef*[28,46] or *vpr*.[47] Most disturbing was that the virulent viruses isolated from the vaccinees had arisen *de novo* as evidenced by their DNA sequences which were unique and distinct from wild-type virus. Once a vaccine virus has been administered, its molecular evolution in vaccinees is beyond human control.

The need for attenuated retroviruses to integrate into host DNA poses the danger of insertional mutagenesis.[48] An adult macaque developed disseminated lymphoproliferative disease 29 months after infection with attenuated, *nef*-deleted SIV.[49] Molecular analysis of its tumor tissues may reveal a role of the vaccine virus in neoplastic transformation.

Attenutated live retroviruses could be transmissible. Ho and Cao[50] report a female long-term survivor of HIV-1 infection whose virus appeared attenuated. This woman was diagnosed initially because she delivered an HIV-1-infected child, who died at age 12 of AIDS. This case raises the possibility that attenuated HIV-1 can be transmitted vertically and is more pathogenic in children than in adults. Alternatively, an attenuated virus could have mutated to a more virulent form in the child.

Live attenuated SIV vaccines seem to require relatively long time periods to induce protective responses. Only macaques challenged with wild-type SIV at 79 weeks post-vaccination with *nef*-deleted mutants were protected, but not those challenged at 8 or 20 weeks.[40] Almond *et al*[80] challenged their macaques at 39 and 49 weeks post-vaccination. Slow generation of protective responses could be dangerous in human vaccinees who could engage in more risk behavior due to a false sense of security, thus increasing HIV-1 exposure.[7,51]

We conclude that the live attenuated virus prototypes which are deleted in the *nef* and *vpr* genes have retained virulence. The success of vaccine strategies using these replication-impaired, live retroviruses is based on host defenses winning the race against vaccine virus replication. We consider their use as candidate human vaccines dangerous because disturbances in the host–virus interaction could tilt the balance in favor of the virus. Such viruses may still be

pathogenic even in adults, if either the rate of virus replication is accelerated or if host immune responses are compromised, since both mechanisms could lead to run-away vaccine virus replication exceeding the threshold. Long term safety studies in co-infected and immunocompromised animals are needed to address these issues. Meanwhile, research with these viruses must go forward to investigate the correlates of protection. Even if these agents are ultimately unsafe as human anti-HIV-1 vaccines, important insights into the mechanisms of resistance to infection with wild-type virus can be gained. After the protective principles are identified, safer vaccine strategies can then be tested for their ability to induce the same host responses.

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SIV pathogenesis during ontogeny: the influence of host factors

SIVΔ3, a mutant of the simian immunodeficiency virus (SIV) that has large deletions in *nef, vpr* and in the negative regulatory element (NRE) of the long terminal repeat (LTR) [1], replicated to high levels and caused lethal AIDS in orally infected macaque neonates [2]. In contrast, intravenously (iv) inoculated adults had low virus loads and remained healthy [2, 3]. To explain this difference in pathogenicity, we introduced the threshold hypothesis [2, 4], according to which retroviral disease only becomes apparent after the virus replicates above a certain threshold in a given host. If virus replication is restrained by any mechanism(s) and does not exceed this threshold, disease will not ensue, even if the virus genome encodes the gene(s) for virulence. We postulated that in mucosally infected macaque neonates, replication of SIVΔ3 was unrestricted and exceeded the threshold, whereas host factors limited replication in the iv-infected adults.

To identify the nature of such host factors, the following parameters were examined systematically in adult and neonatal macaques: a) possible co-infection with other simian pathogens known to induce acquired immune deficiency; b) the route of SIVΔ3 inoculation (iv versus oral); c) the portal of virus entry and early virus target cells; d) antibody responses to homologous and heterologous virus; and e) the role of cellular immune responses. We conclude that differences in cellular immune responsiveness between adults and neonates are the most likely cause for the differential pathogenicity of SIVΔ3.

Results

Ruling out co-infection with simian retrovirus type D serotypes 1, 2, and 3

Could the striking difference in disease development in our neonatal and adult experimental macaques be due to an unrecognized infection with other simian pathogens, rather than be caused by SIVΔ3 itself? Among the many potential known and unknown pathogens that could infect monkeys, exogenous simian retrovirus type D (SRV/D) infection is of con-

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cern. SRV/D comprises five neutralization serotypes, three of which, SRV-1, SRV-2, and SRV-3, also known as Mason-Pfizer monkey virus, have been molecularly cloned and sequenced [5-11]. This group of viruses is prevalent in wild as well as in colony-bred macaques and can cause a clinical syndrome of acquired immunodeficiency that is indistinguishable from SIV-induced disease. While several serological assays have been established to document SRV/D infection, many animals can harbor the virus in the absence of seroconversion. SRV/D isolation in cell culture systems is available, but the 6 week long assay period can be impractical. Serial blood samples from our experimental animals had tested negative for SRV/D antibodies [2].

To further rule out SRV/D infection, we established a DNA polymerase chain reaction (PCR) assay, using primers from conserved regions of the *env* gene of SRV/D serotypes 1, 2 and 3 (Liska, Lerche and Ruprecht, unpublished data; based on the origin of our pregnant macaques, infection with serotypes 4 or 5 is unlikely). One set of nested primers allows detection of all three serotypes and distinguishes SRV-2 from the other two serotypes. A single proviral copy of SRV/D can be detected in 150,000 to 210,000 macaque peripheral blood mononuclear cells (PBMC). When evaluated with a panel of test samples, the assay was 100% specific and 86% sensitive.

Serial DNA samples isolated from the four SIVΔ3-infected infant macaques and from their mothers [2] were subjected to a blinded re-analysis for SRV/D proviral sequences. None were detected. Given the high SIVΔ3 proviral load in the infected infant macaques [2], SIVΔ3 would be at least 20,000 times more prevalent than SRV/D if the latter were present at a concentration that is not detectable by this sensitive PCR assay. Given these new data, together with the repeated negative serological testing, SRV/D infection in our experimental animals is improbable.

Oral versus intravenous SIVΔ3 inoculation

Was the differential SIVΔ3 pathogenicity in orally exposed neonates and iv-inoculated adults due to the route of virus entry? To address this issue, neonate number 95-11 (table I) was administered orally approximately 300 times the virus dose used in the earlier vaccine experiments in adult macaques [3], which was the same dose that led to a 100% infection in our earlier series of neonatal animals [2]. Infant 95-10 received the same virus dose iv. As controls, the mothers of both infants also received the same virus inocula iv (table I). All four animals became infected as measured by positive virus isolation experiments. While a 12 week observation period was too short to assess development of AIDS, the two infected infants already showed abnormalities in the

Table I. Oral or intravenous route of infection of neonatal and adult macaques with cell-free SIVΔ3.

Animal (infant)	*Route*	*Viral status*	*Clinical observation*	*Weeks pi*
95-11	po	Infected	Borderline low $CD4^+CD29^+$ T cells	12
95-10	iv	Infected	Low $CD4^+CD29^+$ T cells	12
N269 (95-10)	iv	Infected	Healthy	12
6543 (95-11)	iv	Infected	Healthy, normal $CD4^+$ and $CD4^+CD29^+$ T cells, inverted CD4/CD8 ratios, even in pre-bleed	12

Two macaque mother/infant pairs were exposed to SIVΔ3 via the routes indicated. The oral virus inoculum was approximately 300 times higher than the dose given intravenously (iv) to adult macaques by Wyand et al [3] (2,000 50% tissue culture infectious doses or $TCID_{50}$). Viral status and clinical observation from the 12 week post-inoculation (pi) time point are shown. po: orally.

Table II. Oral and intravenous exposure of neonatal macaques to SIVΔ3-positive blood.

Infant	*Virus source*	*Route*	*Viral status*	*Clinical observation*	*Age*
94-1	Donor (cell-free SIVΔ3)	po	Plasma: 0; PBMC: 9766	Low $CD4^+$ and $CD4^+CD29^+$ T cells, thrombocytopenia	18 months
95-1	2.5 mL blood	po	Not infected	Healthy	23 weeks
95-2	2.5 mL blood	iv	Plasma +; PBMC +	$CD4^+$ T cells low for age, low $CD4^+CD29^+$ T cells, CD4/CD8 ratio inverted, oral abscess	23 weeks
94-2	Donor (cell-free SIVΔ3)	po	Plasma: 0; PBMC: 9766	Relative loss of anti-Gag ab, borderline low $CD4^+CD29^+$ T cells	18 months
95-6	2.5 mL blood	po	Not infected	Healthy	19 weeks
95-5	2.5 mL blood	iv	High virus load in plasma and PBMC	Low $CD4^+$ and $CD4^+CD29^+$ T cells, inverted CD4/CD8 ratios	19 weeks

SIVΔ3-infected blood from donor macaque infants 94-1 and 94-2 was administered orally (po) or intraveinously (iv) to recipient neonates. At the time of adoptive transfer, neither donor infant had plasma viremia. The virus load in the donor PBMC was measured by end-point dilution by cocultivation in CEMx174 cells; a minimum of 9766 PBMC was required to yield p27 Gag-positive supernatants. ab: antibody.

$CD4^+CD29^+$ T-cell subset. The two infected adults revealed no such abnormalities. It should be noted that parturient rhesus monkeys have been observed to have slightly inverted CD4/CD8 ratios, as was noticed already in the blood sample collected prior to virus inoculation (pre-bleed) of animal 6543. Following infection with SIVΔ3, this animal had no significant changes in its T-cell population.

In another set of experiments, neonatal rhesus macaques were given SIVΔ3-infected blood from infants enrolled in the earlier experimental series [2] (tables II and III). The first donor was infant 94-1, which had AIDS and thrombocytopenia. The viral load in the donor peripheral blood mononuclear cell (PBMC) fraction was high, but plasma viremia was not detected at the time of blood transfer. One recipient infant, 95-1, received an aliquot of 2.5 mL of blood orally, whereas infant 95-2 received the same amount of blood iv. The orally exposed infant did not become infected. The iv-exposed infant, on the other hand, had a rapid decline in $CD4^+$ and $CD4^+CD29^+$ T cells, an inverted CD4/CD8 ratio, and developed a *Staphylococcus aureus* oral abscess. The second donor infant, 94-2, had only developed mild signs of immunodeficiency at the time of blood transfer. Its viral load was equal to that of donor 94-1, and no plasma viremia was detected. Infant 95-6 was exposed orally, and infant 95-5 was exposed iv. Only the latter became infected. In contrast to the donor infant, recipient 95-5 rapidly developed depletion of the $CD4^+$ and $CD4^+CD29^+$ T-cell subsets (table II). The four mothers of the recipient infants were inoculated iv with an equal amount of infected donor blood as their infants. All adults became infected and developed initial peaks of viremia, followed by containment of virus replication. Currently, these animals have very low virus loads in the PBMC fraction, and no plasma viremia can be detected. All four adults have remained healthy, and none have developed abnormalities in the $CD4^+$ T-cell subsets (abnormalities in the pre-bleed may be related to gestation and delivery).

In summary, rhesus macaque neonates infected iv with either cell-free or cell-associated SIVΔ3 have developed signs of disease, even after relatively short periods of observation. In contrast, all iv-infected adults have remained healthy and have been able to suppress virus replication after the initial peaks of viremia. Because the infants exposed orally to infected blood did not get infected, in contrast to the recipient infants given the virus iv, we conclude that the virus dose was relatively low. Nevertheless, T-cell depletion was already observed in recipient infants 95-2 and 95-5. These data indicate that the virus dose also is

Table III. Intravenous infection of adult macaques with SIVΔ3-positive blood.

Mother (infant)	*Source of blood (2.5 mL)*	*Viral status*	*Clinical observation*	*Week pi*
6VJ (95-1)	94-1	Initial peak of viremia, now low virus load in PBMC only	Healthy	23
AA2 (95-2)	94-1	Initial peak of viremia, now low virus load in PBMC only	Healthy, (pre-bleed: $CD4^+$ T cells = 296; now normal)	23
96H (95-5)	94-2	Initial peak of viremia, now low virus load in PBMC only	Healthy, minimally inverted CD4/CD8 ratios, even in pre-bleed	19
NVG (95-6)	94-2	Initial peak of viremia, now low virus load in PBMC only	Healthy, normal $CD4^+$ T cells, inverted CD4/CD8 ratios, absolute lymphocytosis	19

The mothers of the recipient macaque infants listed in table II were exposed iv to the same aliquots of infected donor blood as their infants. pi: post-inoculation.

not responsible for the differential pathogenicity in adults and neonates.

Humoral immune responses in adult and neonatal macaques infected with SIVΔ3

We examined whether neutralizing or infection-enhancing antibody responses to SIVΔ3 infection in rhesus monkeys were associated with attenuation in adults or virulence in infants. Determination of neutralizing antibody titers against SIV_{mac251} grown in H9 cells revealed that infection with SIVΔ3 induces high-titer neutralizing antibodies in infants (fig 1A) as well as in adult macaques (fig 1B and data not shown). These antibodies failed to correlate with virus clearance in the adult and they did not predict slow or rapid disease progression in the infants. When measured against SIV_{mac239} *nef* open, which is the parental virus of SIVΔ3 and a molecular clone derived from the biological isolate SIV_{mac251}, no neutralization could be detected, even when plasmas of infected adults or infants were used at dilutions of 1:2. Thus, the parental SIV_{mac239} proved to be neutralization resistant. Taken together, these data make it implausible that neutralizing antibodies are responsible for the differential SIVΔ3 pathogenicity in adults and neonates.

Next, we tested the same plasma samples for infection-enhancing antibodies. Complement-mediated antibody-dependent enhancement (C′-ADE) of parental SIV_{mac239} infection was measured in MT-2 cells with complement-restored, heat-inactivated plasma samples as described previously [12]. Plasma samples from adult rhesus macaques infected with live, attenuated SIVΔ*nef* for approximately 2 years have been shown previously to be susceptible to C′-ADE antibodies when tested with SIV_{mac251} [12]. Our analysis revealed that the parental SIV_{mac239} is sensitive to C′-ADE using plasma derived from both adults and infants (not shown). Because the C′-ADE responses in infants and adults were similar in both titers and potency, antibody-mediated enhancement of infection is an unlikely explanation for the differential SIVΔ3 virulence in adults and infants.

The putative role of cellular immunity in controlling virus load and virulence

After systematically analyzing viral and host factors that might explain the differential effects of SIVΔ3 in adults and infants, we conclude that age at virus exposure most strongly predicts an adverse outcome. Because neither neutralizing nor infection-enhancing antibody responses can explain our results, two variables remain, ie, an intrinsic difference in the ability of neonatal cells to support replication of an SIV mutant deleted in *nef*, *vpr* and NRE, or, alternatively, a crucial influence of host-cellular immune responses. Currently, we are testing both factors.

A number of other viral infections have been found to take a more aggressive course in neonates as compared to adults. For instance, herpes simplex virus type 1 or 2 infection in

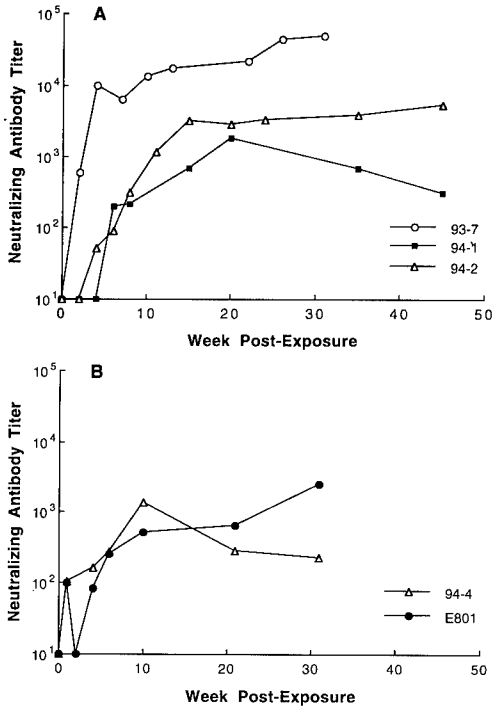

Fig 1. Neutralization of SIV_{mac251} by plasma samples from adult and infant macaques infected with SIVΔ3. SIV_{mac251} was grown in H9 cells and tested for neutralization in CEMx174 cells. Briefly, all plasma samples from sterile, preservative-free heparinized blood were heat-inactivated (56 °C, 1 h). Cell-free virus (50 µL containing 0.5-1 ng of p27) was added to multiple dilutions of test plasmas (100 µL) in triplicate wells of 96-well plates and incubated at 37 °C for 30 min before addition of 10^5 CEMx174 cells/well. Cell density was reduced and medium was replaced 3 days later. Infection caused extensive syncytium formation and cell killing in approximately 6 days in the absence of antibody. Neutralization was measured by staining with Finter's neutral red in poly-L-lysine-coated plates as described [13]. Assays were harvested when virus-induced cell-killing in untreated, infected control wells was > 70% but < 100%. Neutralization titers are given as the reciprocal dilution required to protect at least 50% of cells from virus-induced cell killing. (**A**) Macaques exposed orally at birth to SIVΔ3. (**B**) Mother (E801)/infant (94-4) pair exposed orally or iv to SIVΔ3-infected blood from infant 93-7. At the time of adoptive transfer, the donor infant had AIDS. These animals have been described previously [2].

immunocompetent adults causes only localized disease. In contrast, infection in neonates and immunocompromised adults is widespread and can have fatal consequences. Given these clinical observations in other systems, we favor cellular immune responses as the dominant determinant for SIVΔ3 virulence in neonatal macaques. We hypothesize that effective antiviral cellular immune responses, which can be generated by adult but not neonatal macaques, allow the adult animal to reduce virus replication to a trickle, thus preventing the virus load to reach the threshold. Consequently, the underlying virulence of SIVΔ3 is not revealed in immunocompetent adults.

If attenuation of SIVΔ3 or other *nef*-minus mutants in adult macaques can be ascribed to cellular immune responses, can live, attenuated retroviruses deleted in *nef* be used as anti-AIDS vaccines in adults? Our data in neonatal macaques raise serious safety concerns. Because this virus induced lethal AIDS, SIVΔ3 can be regarded only as attenuated in its ability to replicate, but not in its ability to cause disease. Consequently, factors that can overcome the relative restriction of virus replication could allow the virus to exceed the putative threshold, even in adults. We hypothesize that any individual, adult or infant, has an inherent threshold of virus replication that must be exceeded before retroviral virulence can become apparent. If this threshold is not reached, disease will not develop, even if the virus encodes the necessary genes to induce disease. Because infection with *nef*-deleted viruses persists for the life of the host, temporary loss of cellular immune competence due to intercurrent illness, or permanent loss due to aging, could result in an increased level of virus replication. Under such circumstances, disease could develop even in adults. As long as no data are available on the safety of *nef*-deleted, attenuated live viruses in immunocompromised or co-infected adults, serious safety concerns remain.

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Short Communication

Simultaneous Detection of Simian Retrovirus Type D Serotypes 1, 2 and 3 by Polymerase Chain Reaction.

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ABSTRACT

Asymptomatic infection of macaques with simian retroviruses type D (SRV/D), the etiologic agents of one form of retrovirus-induced simian immunodeficiency disease, can confound experiments with the simian immunodeficiency virus (SIV), which also induces immunodeficiency disease in macaques. The SIV/macaque model is the preferred non-human primate model for AIDS-related research. Serological screening for SRV/D alone is insufficient because not all infected animals seroconvert, and virus isolation by co-cultivation may require four to six weeks. We have established a DNA polymerase chain reaction (PCR) assay. One set of nested primers allows detection of SRV/D serotypes 1, 2 and 3 and distinguishes SRV-2 from the other two serotypes. The PCR assay is sensitive; a single proviral copy of SRV/D could be detected in 150,000 to 210,000 macaque peripheral blood mononuclear cells (PBMC). When applied to a panel of virus isolation-positive macaque samples, the PCR assay was positive in 100% of the tests. No false positive results were seen when known specific-pathogen-free (SPF) macaques were examined. We propose that macaques be screened with a combination of SRV/D serology and this DNA PCR assay prior to enrollment into experiments with SIV.

Infection with simian type D retroviruses (SRV/D) is prevalent in wild as well as in colony-born macaques[1-3]. Five neutralization types have been identified[3], three of which, SRV-1, SRV-2 and Mason-Pfizer monkey virus (MPMV, SRV-3), have been molecularly cloned and sequenced[4-6].

Macaque models provide an opportunity to develop vaccines against simian immunodeficiency virus (SIV), which induces a disease similar to human AIDS. SIV experiments in macaques can be affected adversely by inapparent SRV/D infection. Tests to screen for SRV/D include enzyme-linked immunosorbent assays (ELISA) for antibody or antigen[7,8], fluorescent antibody assays[9], and Western blot analysis (WB)[8]. Overall, SRV/D seroprevalence ranges from 1-4%, but may be much higher in some hyper-endemic macaque colonies. Indeterminate SRV/D serological results, characterized by reactivity only to the products of a single viral gene on Western blot (WB), can be as high as 30%[10]. For these reasons and because of the existence of seronegative SRV/D carriers[11], virus isolation by co-cultivation is performed[12]. We have developed a DNA PCR assay to detect a conserved nucleotide sequence in the *env* genes of SRV/D serotypes 1-3.

Heparinized blood samples originated from 15 cynomolgus monkeys (*Macaca fascicularis*) collected during an outbreak of SRV/D, type 2 in colony (California Primate Research Center, Davis, CA), and 7 specific pathogen-free (SPF) rhesus monkeys (*Macaca mulatta*) (MD Anderson Cancer Center, Bastrop, TX). Genomic DNA from macaque PBMC, Raji cells infected with SRV/D serotypes 1 (R-20216), 2 (R-10867), or 3 (R-23200), and CEMx174 cells was isolated as described[13]. Plasmid DNA containing cloned SRV-1 (pSRV-1)[4], SRV-2 (D2C/Oregon)[14], and SRV-3 (pSHRM15)[15], respectively, was isolated using QIAGEN plasmid kits (QIAGEN, Chatsworth, CA). For titrations, 10-fold dilutions of plasmid DNA (1×10^{-1} to 1×10^{-10} ng) were prepared in the presence of 1 μg CEMx174 DNA.

Sequences of external primers (SRV*env*1E: 5′ GCC CGT GGT AAA GAA AAA ATT G 3′; SRV*env*2E: 5′ ACT TCT GCT AGA GAG TCT AC 3′) and nested primers (SRV*env*3N: 5′ GAA GAT TGC TGG CTG TGC TT 3′; SRV*env*4N: 5′ ATA GCT GGA ATG GGG ACA GG 3′) were derived from the *env* genes of SRV/D serotypes 1-3[4-6] (NCBI-GenBank, Bethesda, MD; Accession # M11841, M16605 and M12349, respectively). The primer sequences are located at the following positions: SRV*env*1E: 6,500-6,522 (type 1), 6,468-6,490 (type 2), and 6,884-6,906 (type 3); SRV*env*2E: 7,208-7,228 (type 1), 7,149-7,169 (type 2), and 7,589-7,609 (type 3); SRV*env*3N: 6,593-6,613 (type 1), 6,558-6,578 (type 2), and 6,974-6,994 (type 3); and SRV*env*4N: 6,995-7,015 (type 1), 6,936-6,956 (type 2), and 7,376-7,396 (type 3). Corresponding sequences of SRV/D (type 1 and 3) served for designing of both sets of SRV/D-specific primers. All DNA samples were analyzed for amplifiable cellular DNA with primers for human β-actin[16] (XAHR 17 and XAHR 20, Research Genetics, Huntsville, AL). Bacteriophage λ DNA and primers PCO1 and PCO2 (GeneAmp PCR Reagent Kit, Perkin-Elmer Cetus, Branchburg, NJ) were used as control.

Reaction conditions for both sets of SRV/D-specific primers consisted of 10 mM Tris-HCl, pH 9.2; 1.5 mM $MgCl_2$; 25 mM KCl (Opti-Prime™ buffer #9; Stratagene, La Jolla, CA); 100 μmol of each dNTP (Amplitaq, Perkin-Elmer Cetus); 50 pmol of each primer (Research Genetics) and 1.25 U of *Taq* polymerase (Amplitaq, Perkin-Elmer Cetus) in 50 μl. Typically, 1 μg of macaque genomic DNA was analyzed. For nested PCR, 1 μl of product from the first PCR round was added to 50 μl of the second round reaction mixture. Alternatively, "hot start" PCR, in which *Taq* polymerase was inhibited with TaqStart™ antibody (Clontech, Palo Alto, CA), was used. Reaction mixtures were overlayed with 20 μl of mineral oil (Sigma). PCR was performed in a Perkin-Elmer GeneAmp PCR System 9600 (Perkin-Elmer Cetus, Norwalk, CT). Cycling conditions for the first PCR round were: initial denaturation (98°C, 15 sec) followed by 6 cycles of "touchdown"[17] amplification, which started with a melting step

(97.5°C, 15 sec), annealing (58°C, 40 sec) and extension (72°C, 55 sec). In each subsequent cycle, the melting temperature was lowered by 0.5°C, but melting times remained constant; the annealing temperature was lowered by 1°C, and the annealing time was shortened by 5 sec; extension temperatures stayed constant but the extension time was shortened by 5 sec. Then, cycling continued (94°C for 15 sec; 53°C for 15 sec; 72°C for 30 sec) for 40 cycles, and was finished at 72°C for 6 min. Nested PCR began with denaturation (94°C, 1 min) followed by 40 cycles of annealing (55°C, 15 sec), extension (72°C, 30 sec) and denaturation (94°C, 15 sec) per cycle and terminal extension (72°C, 6 min). Negative controls consisted of genomic CEMx174 and uninfected Raji cell DNA, H_2O without added template, and CEMx174 cell DNA without *Taq* polymerase. PCR products were analyzed by agarose gel electrophoresis.

Antibodies to SRV/D in macaque serum were measured by enzyme immunoassay (EIA), and EIA-reactive sera were confirmed by WB as previously described[10]. The EIA result were considered positive if the O.D. was $\geq$2.5-times value of negative controls. Results for WB were considered positive if reactivity against the product of 2 viral genes were observed. Blots showing reactivity against product of a single viral gene were considered "indeterminate".

SRV/D isolations were performed by co-cultivation of macaque PBMC (2×10^6/ml) with the permissive cell line Sup-T1, as previously described[10]. Briefly, macaque PBMC were separated from heparinized whole blood by Ficoll gradient centrifugation. The PBMC suspensions of 1×10^6 cells/ml were stimulated with *Staphylococcus* enterotoxin A (SEA) for 48-72 hours, then co-cultivated with an equal volume of Sup-T-1 cells at a concentration of 5×10^5 cells/ml. Levels of reverse transcriptase (RT) in culture supernatants were monitored at regular intervals for six weeks. Cultures were considered positive if RT levels were $\geq$5x background in three samples.

Our PCR assay could amplify *env* sequences of SRV/D serotypes 1-3 and allowed the distinction of SRV/D serotype 2 from the other two (Fig. 1A, 1B). Plasmids or genomic DNA from infected cells could be amplified equally well (Fig. 1A,B and 2A,B). Even though primers SRV*env*3N and SRV*env*4N displayed 4 or 3 mismatches, respectively, when compared to SRV-2 *env* (Table 1), target DNA could be amplified effectively. The PCR sensitivity was determined by end-point titration of cloned SRV/D in the presence of carrier DNA. The first PCR round detected of 1×10^{-4} ng to 1×10^{-5} ng of plasmid DNA representing 8.5×10^{3} (SRV-1), 8.6×10^{2} (SRV-2), and 6.9×10^{2} (SRV-3) proviral copies (Fig. 1A). The first PCR round became more sensitive using "hot start" PCR[18] (not shown). The second PCR round could detect 1×10^{-8} ng of plasmid DNA (Fig. 1B), which represents from 0.7 to 0.9 proviral copies in 150,000 cells.

Genomic DNAs from 15 macaques infected with SRV/D (type 2), and 7 DNAs from SPF macaques were screened by PCR, and the results were compared to those obtained by EIA, WB and virus-isolation monitored by RT (Table 2). All cellular DNA samples analyzed were amplifiable as evidenced by β-actin-specific PCR (not shown). The PCR results correlated well with those obtained by virus isolation. Fifteen of 15 macaque samples shown to be RT-positive were PCR positive (Tab. 1 and Fig. 3B). By simultaneous serological screening, only one animal was positive and 3 others had indeterminate WB (Tab 1). All DNA samples from 7 SPF monkeys were PCR negative (Fig. 2A,B). Taken together, these results indicate a sensitivity of 100% and a specificity of 100%.

In sum, we have developed a sensitive DNA PCR assay to detect proviral DNA of SRV/D serotypes 1-3, using conserved *env* sequences, which allowed detection of a single proviral copy in 150,000-210,000 cells. Apparently, the internal mismatches (none of them located within last 5 bases of 3′ oligomer ends) of the nested primers with SRV-2 DNA did not affect the yield of PCR product. SRV/D outbreak in UC Davis cynomolgus monkeys was

due to SRV-2. Our assay was 100% accurate in identifying infected samples. These results agree with those of Kwok et al.[19] who analyzed the effects of various primer-template mismatches on DNA amplification. The fact that 14 RT-positive macaques were seronegative underscores that antibody testing alone is insufficient to detect all infected animals. Of these, 14 animals were PCR positive.

Co-cultivation of rhesus PBMC and sequential screening for virus-associated RT is effective in identifying retrovirus-infected animals, but a 6-week wait for results can be problematic[10]. SRV/D-specific PCR analysis, using either generic or serotype-specific *gag* primers followed by hybridization with radiolabeled oligoprobes, was employed previously to search genomic human PBMC DNA for the presence of SRV/D sequences[20]. This assay could distinguish serotypes 1 and 3 from serotype 2. A recently published PCR approach for SRV/D DNA detection[21] used 3 different sets of primers specific for each serotype and generic radiolabeled DNA probes. The sensitivity of this assay, although appearing significantly lower, has not been evaluated fully. Our PCR assay uses one set of nested primers for the simultaneous detection of all three serotypes and does not depend on the use of radiolabeled probes. Furthermore, this assay is quantitative since plasmid DNA for each serotype is titrated. Combined with serological testing, PCR represents a rapid, sensitive and reliable diagnostic tool for colony management and for laboratories using macaque models to study AIDS-related viruses.

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TABLE 1. NESTED PRIMER AND SRV-2 DNA SEQUENCES

Primer name	*Virus*	*Primer* [a] *and/or viral sequence* [b]	
SRV*env*3N	SRV-1	5′ GAA GAT TGC TGG CTG TGC TT 3′	a,b
	SRV-2	a.c ..g ... .a	b
	SRV-3	... ...	a,b
SRV*env*4N	SRV-1	ATA GCT GGA ATG GGG ACA GG	a,b
	SRV-2	... ... .ca ..t	b
	SRV-3	... ...	a,b

LEGENDS

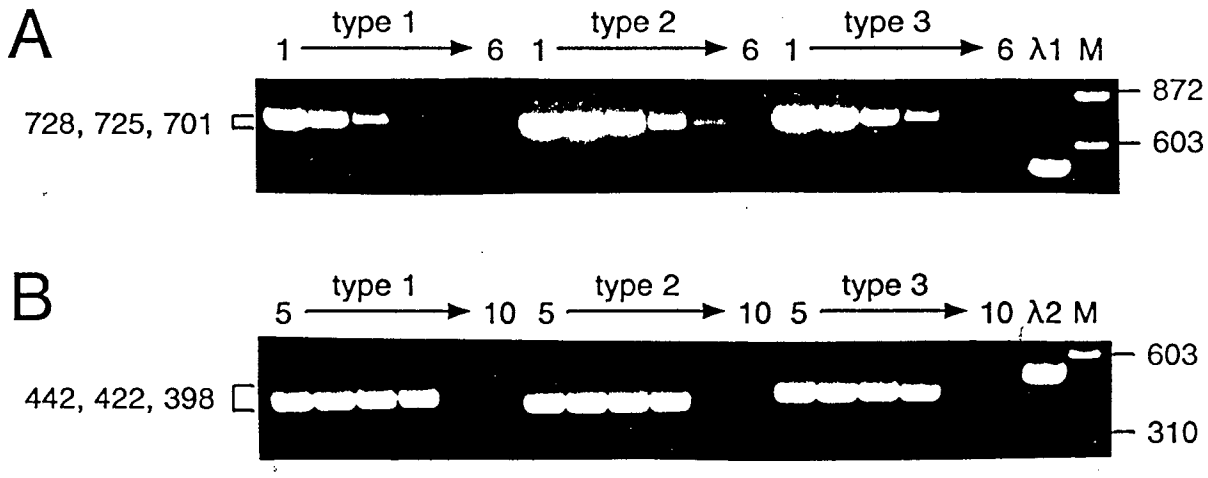
TABLE 1. The primer sequences listed above were derived from identical DNA sequences of SRV-1 and SRV-3, respectively. Internal mismatches of the primers, when compared to the SRV-2 DNA sequence, are noted in lower case. Because the mismatches were not located close to the 3′ end of the nested primers, the sensitivity of the second round of the PCR assay was not affected when tested with plasmid DNA encoding SRV-2 (see Text). The outer primers, used in the first round of the PCR assay, had no mismatches.

TABLE 2. Sex, age, clinical status, serology, virus isolation and PCR (env) results of cynomolgus monkeys infected naturally with SRV/D, type 2. All animals were infected during an outbreak of SRV/D, type 2, in the colony. Thus, the exact time of infection for each animal is unknown. Following recognition of early clinical cases, the entire colony was screened. Many animals were found to be infected without clinically apparent disease. Serology: IND, indeterminate pattern on Western blot.

FIG. 1. Titrations of SRV/D, serotypes 1-3. Ten-fold dilutions of plasmid DNA (1×10^{-1} to 1×10^{-10} ng, lanes 1-10) were amplified in the presence of 1 μg of CEMx174 DNA. λ_1 and λ_2, independent PCR controls (Perkin-Elmer Cetus). Amplified products were resolved on 1.5 % agarose gels and stained with ethidium bromide. (A) The first PCR round of cloned SRV/D plasmid DNAs yielded specific products of 728 bp (type 1), 701 bp (type 2), and 725 bp (type 3), respectively. (B) The second (nested) PCR round yielded specific bands of 422 bp (SRV-1), 398 bp (SRV-2), and 442 bp (SRV-3). The last positive dilution of plasmid DNA (1×10^{-8}, types 1-3) is equal to one proviral copy in 150,000 - 210,000 cells.

FIG. 2. Controls and standards. First (A) and second (B) PCR rounds are shown of genomic DNA from 7 SPF Rhesus Monkeys (S1-S7), CEMx174 cell DNA (C), uninfected Raji cell DNA (Rj), H_2O without added template (W), and CEMx174 cell DNA without *Taq* polymerase (T-). Raji cells infected with SRV1-3 (lanes R1-R3) were used as positive controls. Agarose gels (1.5%) were stained with ethidium bromide. Marker: ΦX174 DNA digested with *Hae*III.

FIG. 3. Screening of 15 cynomolgus monkey DNA samples (lanes 1-15) for the presence of specific proviral SRV/D (type 2) DNA in the first (A) and second (B) PCR round. Raji cells infected with SRV/D, types 1-3 (lanes R1-R3), were used as positive controls. Agarose gels (1.5%) were stained with ethidium bromide. Marker: ΦX174 DNA digested with *Hae*III.


A
type 1
type 2
type 3
1
6
1
6
1
6
λ1
M
728, 725, 701
872
603
B
type 1
type 2
type 3
5
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5
10
5
10
λ2
M
442, 422, 398
603
310

Figure 1

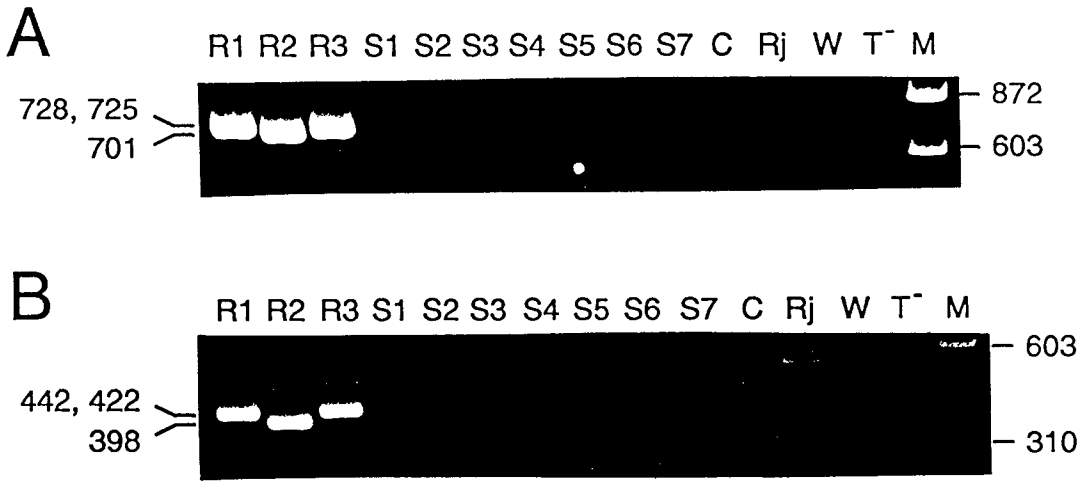
A
R1 R2 R3 S1 S2 S3 S4 S5 S6 S7 C Rj W T⁻ M
728, 725
701
872
603
B
R1 R2 R3 S1 S2 S3 S4 S5 S6 S7 C Rj W T⁻ M
442, 422
398
603
310

Figure 2

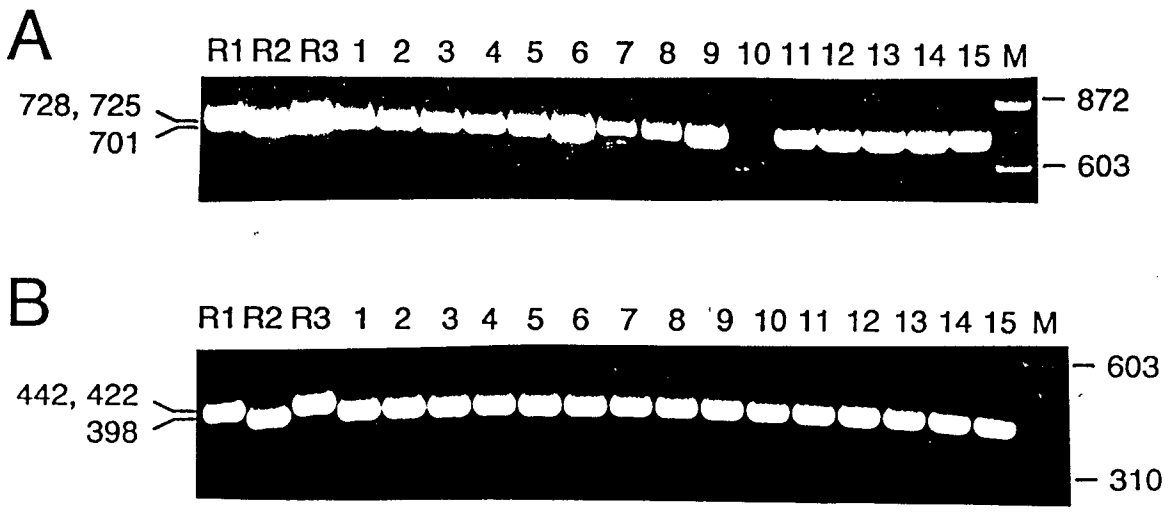
A
R1 R2 R3 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 M
728, 725
701
872
603
B
R1 R2 R3 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 M
442, 422
398
603
310

Figure 3

Induction of Protective Immunity by Exposure to Low Doses of a Live, Pathogenic Retrovirus

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SUMMARY

We have proposed earlier that retroviral disease can only become apparent if the viral load exceeds a certain threshold in a given host. Subthreshold replication will not lead to disease, even if the virus is replication competent and encodes the genes for virulence, because host immunity either clears all virus-infected cells (transient infection), removes all cells actively expressing proviral sequences (latency), or restricts virus replication to low levels (chronic non-progressive infection). Using a mouse model system where inoculation with a potentially lethal dose of Rauscher murine leukemia virus (RLV) followed by anti-viral drug treatment induces protective cellular immunity, PCR anaylsis suggests that this immune response is able to clear a transient viral infection. Host RLV-specific $CD8^+$ CTL responses coincide with viral clearance in these animals. Evidence to suggest that cellular immunity functions in a similar manner in animals receiving no anti-viral drug prophylaxis was provided by observing both a lower degree of overall survival in cyclosporin-treated, RLV-infected mice and by inoculation of T cell-deficient nude mice, where it was found that a stock of RLV is ten-fold more infectious than in their normal, isogenic counterparts. Furthermore, when immunocompetent mice that cleared RLV three weeks after inoculation with various low doses of live pathogenic RLV were re-challenged with 20 or 200 50% animal infectious doses, 30% of the mice remained virus free after high-dose virus rechallenge. These data demonstrate that host immunity can not only clear sub-threshold doses of a pathogenic retrovirus, but also protect against subsequent higher virus challenge.

INTRODUCTION

We have suggested earlier that following retrovirus infection within a host animal, a threshold level of viral load determines whether pathogenesis will occur (Baba et al., 1995) . No disease will occur if virus replication, and overall viral burden, is maintained below such a threshold, irregardless of whether the retrovirus encodes the genes necessary for virulence. On the other hand, once this threshold level of virus load is breached, pathogenesis will ensue. Host immune responses may operate to keep virus replication below threshold by: 1) clearing all virus infected cells, resulting in transient infection; 2) clearing all cells actively expressing provirus, thereby inducing latency or; 3) inducing a state of chronic non-progressive infection by limiting virus replication to low levels.

Evidence suggests that, indeed, host factors play a major role in maintaining viral loads below threshold levels. For example, it was found that inoculation of adult macaques with a replication-impaired SIV mutant, deleted in the *nef* and *vpr* genes (SIVΔ3), caused no disease and could induce immune protection against subsequent challenge with wild-type SIV (Daniel et al. 1992; Wyand et al. 1996). However, we demonstrated that SIVΔ3 itself was pathogenic in neonatal macaques which were orally infected with the mutant virus (Baba et al. 1995). These observed differences in pathogenesis between adults and neonates induced by SIVΔ3 do not appear to be related to any changes in the innate virulence of the virus, dosage, or route of infection, nor does humoral immunity appear to play a role (Baba et al. 1995; 1996). As such, it is believed that cellular immunity in the adult hosts, as indicated by the observed protection against subsequent challenge, may have combined with genetic deletions in the mutant virus to keep replication at subthreshold levels and that such an immune response was absent in the neonates.

Our previous studies using the Rauscher murine leukemia virus (RLV) model have provided strong evidence for cell-mediated immunity as a mechanism for clearance of retrovirus infection. It was found that treating mice with a combination of AZT and interferon-α (IFN-α) could prevent viremia following infection with a potentially lethal dose of live, RLV (Ruprecht et al. 1988; Ruprecht et al. 1990a). Post-exposure prophylaxis with this drug combination after several rounds of RLV replication still resulted in no viremia suggesting that the animals were able to ward off low-levels of viral replication (Ruprecht et al. 1990b). Similar experiments in athymic nude mice, whereby only with a 10-fold lower virus inoculum could nude mice be protected, suggested that host cellular immunity was responsible (Ruprecht and Bronson, 1994). Rechallenge experiments in normal mice showed that protective immunity was, indeed, generated by initial exposure to live, pathogenic virus which was made attenuated *in vivo* by drugs which interfered with viral replication (Ruprecht et al. 1990a). Adoptive transfer studies showed that immune serum had little protective effect but demonstrated that immune T cells alone from these animals could confer protection to naive recipient mice (Ruprecht et al. 1990b) and that protection required both $CD4^+$ and $CD8^+$ T cells (Hom et al. 1991).

Many studies have now suggested the importance of virus-specific CTL responses for the control of viremia in persons with HIV-1 infection, especially during the initial stages of primary infection (Carmichael et al. 1993; Barrow et. al. 1994; Koup et al. 1994). In this context, the nature of the protective cellular immune response in mice receiving post-exposure chemoprophylaxis following high-dose RLV inoculation was evaluated and we also tested by PCR anaylsis if transient, low-level infection occured allowing the generation of RLV-specific immunity. In addition, we examined whether exposure to low-dose, pathogenic RLV without anti-viral drug therapy can also induce protective immunity.

RESULTS

CTL activity in RLV inoculated, anti-viral drug treated animals.

The nature of the cellular immunity which plays a key role in preventing viremia in mice inoculated with RLV and receiving anti-viral drug treatment was examined by measuring RLV-specific CTL responses. Mice were injected with a high dose of RLV (5000 50% Animal Infectious Doses; AID_{50}) and treatment with IFN-α and AZT was started 3 hrs later. *Ex vivo* RLV-specific CTL activity in spleen cells from these animals was then measured at various time points after RLV injection. As shown in Figure 1, CTL activity was observed starting on day 11, peaked at day 21 and dropped off rapidly by day 24 post-infection. No CTL activity was present in spleen cells from mice which received the combination of drugs alone.

Negative selection experiments indicated that the bulk of the RLV-specific CTL activity in spleen cells from RLV-inoculated, anti-viral drug-treated mice resided in the $CD8^+$ T cell population (Figure 2). To confirm this, purified $CD4^+$ and $CD8^+$ T cell subsets from identically infected and treated animals wetre tested for cytotoxicity and, as shown in Figure 3, all RLV-specific CTL activity was $CD8^+$ T cell mediated. It should be noted that in some animals RLV-specific CTL responses could not be detected (Figure 1 and 2) and in one case, CTL activity was still seen despite $CD8^+$ T cell depletion.

One group of RLV-infected, drug-treated animals was rechallenged with an acute dose of virus after demonstrating clearance of the initial RLV infection by d21. Interstingly, anly a small RLV-specific ex vivo CTL response was found in the spleen cells of these animals on d1 after challenge (11-12% specific cytotoxicity) and little, if any, CTL activity was seen thereafter (Figure 4).

Temporal clearance of virus infected cells.

To measure the extent of virus infection following inoculation with high dose RLV in conjunction with AZT plus IFN-α drug treatment, semi-quantitative PCR analysis of DNA from splenic isolates using oligonucleotide probes specific for proviral RLV sequences was performed. Since the mouse genome contains multiple copies of integrated retroviral sequences, primers specific for RLV needed to be found. Among several sets of primares derived from env sequences and from the U3 region of the 3' LTR of cloned R-SFFV (pBC10) (Bestwick et al, 1984) tested, those described here were specific for an exogeneous RLV sequence and no signal was seen in genomic DNA obtained from naive BALB/c mice (data not shown).

In most mice injected with RLV, virus-specific sequences were detected immediately following inoculation, even after 15 min (Figure 5). These signals were present in the virus inoculum and probably resulted from cellular DNA contamination. In untreated, infected mice, the band intensity greatly increased beginning on day 4 and continued to do so through the time course of the experiment. In virus-exposed, drug-treated mice, the band intensity also increased on day 4 but began to decrease by day 8. On day 20, either a faint band or no signal at all was observed in drug-treated mice. Numerous negative control DNA samples were included in the analysis; 95% of 60 negative control samples proved to be negative. Thus the faintly positive scoring spleen DNA samples of animals given post-exposure chemopropylaxis are not likely due to random contaminations. It is of interest that the timing of the decrease in band intensity on day 8 precedes the onset of observable CTL activity (day 11) yet peak CTL activity coincides with the disappearance. The findings suggest that viral replication was kept below threshold by drug treatment and allowed a protective CTL immune response to develop which cleared transient viral infection from the animals.

Immunosuppresion following acute virus exposure accelerates disease progression.

To further demonstrate the role of cellular immunity during acute RLV infection, groups of mice were treated with CsA, with vehicle only or left untreated following virus inoculation. CsA-treated mice had a significantly shorter survival as compared to control animals (Figure 6).

Differential apparent infectivity of RLV in normal and immunodeficient animals.

The next series of experiments were designed to ask whether cellular immunity may function to keep viral replication subthreshold if animals are inoculated with a low-dose of RLV in the absence of antiviral drug treatment. To test this, the apparent infectivity of a stock of RLV was compared between normal, immunocompetent BALB/c mice and isogenic, T cell-deficient nude mice. Both sets of animals were inoculated with decreasing concentrations of a stock of RLV and mice were assayed for signs of viremia 21 days later. Serum from individual animals was tested for the presence of RLV antigens by immunoblotting and results were also confirmed by testing serum for the presence of infectious virus by XC-assay. Using these analyses, animals were scored for the presence of virus and the results of a dose titration are shown in Figure 7. In both sets of animals a dose-dependent response was seen and it was found that the AID_{50} in normal mice was approximately a $\log_{10}$ higher than the AID_{50} for BALB/c nude mice. The innate lack of cellular immunity in nude mice strongly suggests that such an immune response functions in the immunocompetent normal mice to prevent replication and clear RLV when it is administered at a relatively low dose.

While demonstrating a difference in the apparent infectivity of a given low dose of RLV between normal and nude mice, the data in Figure 7 showing the infectivity in nude mice also serves as an indication of the presence of live virus in these low dose

inocula. Thus, the finding that 100% of the nude mice became infected if they received inoculations of $\geq$ 0.4 AID_{50} confirmed the presence of live virus in these dosages.

Evidence for protection against lethal challenge with RLV.

It was important to test whether immunization with low dose, live RLV could induce protective immunity in normal, untreated animals. Mice which were determined by immunoblotting and XC assay to be RLV negative following low dose inoculation were challenged iv with acute doses (20 or 200 AID_{50}) of RLV. Animals were then sacrificed 21 days after challenge, the degree of splenomegaly was measured, and serum and spleen cells were analysed for RLV antigens by immunoblot analysis. Partial protection was observed in all groups of animals challenged with 20 AID_{50} (Table I). However, the data in Figure 7 demonstrated that only at the two highest initial doses (0.4 and 2.0 AID_{50}), 100% of the nude mice became infected and it is only at these initial doses could we therefore be assured that low dose inoculation was with live virus. Overall, it was found that, of those animals which definately received live RLV in the initial low dose inocula, 30% became virus free after challenge with lethal doses of RLV. In all cases the presence or absence of virus by immunoblot analysis was confirmed by XC assay (data not shown). Together, these results suggest that vaccination with live retrovirus at sub-acute doses can induce limited immune protection against subsequent challenge with lethal virus doses.

Surprisingly, no *ex vivo* RLV-specific CTL activity was detected at any time following low-dose RLV inoculation (data not shown).

Protection is long term.

Mice which were initially inoculated with low-dose RLV and found to be virus free 21 days after challenge with an acute dose of virus were reanalysed 8 months after challenge for signs of viremia. Serum from each mouse was assayed for RLV antigens and

for infectious virus by XC assay and it was found that among the 4 animals which were virus free after challenge with 200 AID_{50}, 3 remained free of RLV and 4 of 5 animals challenged with 20 AID_{50} were still virus free at this time.

DISCUSSION

In the present study, we have shown that RLV-specific, $CD8^+$ CTL activity has a temporal association with both retrovirus clearance and the establishment of protective immunity in mice inoculated with high-dose RLV and given anti-viral drug therapy. We have also shown that cellular immunity plays an important role in keeping retrovirus replication below disease-inducing threshold levels in low-dose infections with the demonstration that the AID_{50} of a given stock of RLV is approximately 10-fold lower in nude mice compared with that in immunocompetent normal mice. More importantly, it was found that approximately 30% of the immunocompetent mice which cleared a initial exposure to definate live virus (as evidenced by 100% infectivity of the same dose in nude animals) were protected against subsequent challenge with a lethal dose of RLV.

The experimental results presented here are all consistent with the idea that cellular immunity serves as the first line of defense to keep viral loads below threshold levels following retrovirus infection. The threshold hypothesis proposes that if viral loads exceed a pre-determined level, pathogenesis will occur while sub-threshold replication will not lead to disease, even if the virus is fully pathogenic. As to the models we evaluated here, in one case, the anti-viral drug combination of AZT and IFN-α kept RLV replication levels low during the crucial early time points following infection allowing a cellular immune response to develop. In the second case, the aim was to achieve early, sub-threshold immunizing levels of virus by using low-dose RLV inocula which would be kept from exceeding a pathogenic level by immune neutralization. Though the level of protective immunity was certainly more complete when high-dose RLV plus antiviral combination drug therapy was administered, 90-100% (Ruprecht et al. 1990a), our results with low-dose inocula clearly show that immune protection can be induced in the absence of anti-viral drug treatment.

Studies in rhesus macaques to examine the question of whether low-dose inoculation with live, pathogenic SIV can induce immune protection against subsequent

high-dose challenge have also examined virus titration and, in some cases, resistance to high dose challenge was observed (Dittmar et al, 1995; Salvato et al, 1994; Clerici et al, 1994). In those studies, however, it was not possible to distinguish live virus from dead virus particles in the inoculation preparations. By directly comparing the apparent infectivity of a given virus dose in normal mice versus isogenic nude mice, we were able to demonstrate with certainty that the immunocompetent animals received live virus. The comparative experiments performed in this study also allowed us to assess the contribution of host immunity in clearing the virus following the initial low dose inoculations, a mechanism which could not be addressed directly in the primate studies.

In the low-dose inoculation experiments we describe, it is unclear why we were unable to observe direct RLV-specific CTL responses as was seen following high-dose RLV inoculation plus chemoprophylaxis. Experiments are ongoing to assess other immune correlates of the protective immunity achieved in from low-dose inoculation. It is intriguing,however, to speculate that the greater degree of protection observed in the high-dose inoculated mice (>90%, Ruprecht et al. 1990a) may be associated with the high degree of *ex vivo* RLV-specific CTL activity.

The data we have presented shares some common attributes with clinical data regarding HIV-1 infection and immunity, and extends some of the conclusions concerning retrovirus infection. Firstly, among individuals infected with HIV-1, there is now much evidence showing a strong association of CTL activity with the initial control of acute viremia during the primary infection, as well as with long-term nonprogressing infection (Borrow et al. 1994; Carmichael et al. 1993; Klein and van Baelen 1995; Rinaldo et al. 1995; Harrer et al. 1995). In addition, it is now well accepted that HIV-1 specific CTL responses arise early in HIV-1 infected individuals with neutralizing antibodies coming up later (Koup et al. 1994; REFS). Our previous finding of little neutralizing antibody activity (Ruprecht et al., 1990a) and our current finding of a RLV-specific $CD8^+$ CTL response following high-dose RLV infection and post-exposure

anti-viral drug therapy is consistent with these results and suggests that such a response may be involved in the control of viremia in any given retroviral infection. Additionally, although the studies in HIV-1 infected humans show an association with the control of viral replication, the data presented here, along with our previous data showing protection from rechallenge (Ruprecht et al. 1990a; 1990b; Hom et al. 1991), suggest that $CD8^+$ CTL responses in the early stages of retroviral infection are associated with inducing a state of full-fledged protective immunity.

Secondly, the models we have investigated in this study bear a strong resemblence to those cases of HIV-exposed individuals who do not seroconvert or show signs of HIV infection, yet show evidence of HIV-1 specific T cell immunity. These include newborn children of HIV-1 infected mothers, health care workers exposed to HIV-infected blood or body fluids, prostitutes, iv drug users and individuals who have had unprotected sexual intercourse with HIV seropositive individuals (reviewed in Shearer and Clerici, 1995; Rowland-Jones and McMichael, 1995). In those individuals where it appears that long-term HIV-specific immunity, as shown by CTL or Th1-type immune responses, is associated with seronegativity and absence of virus, it has been suggested that these cases may represent instances of a low-dose exposure to HIV-1 or defective virus which was of sufficient magnitude to induce a protective immune response. This would appear to be the case in individuals who have had multiple exposures (REF). However, it cannot be ascertained whether the immune responses observed following a single exposure were truly protective and had cleared a transient, potentially pathogenic retrovirus infection. Our current results indicate, indeed, that a protective cellular immune response is possible following a single low-dose exposure to live retrovirus and may serve as a model to mechanistically confirm that apparent resistance to viral infection may be due to acquired cellular immunity following unrecognized low-does exposure. It will be of importance to determine whether a

stronger degree of protective immunity can be generated to low-dose inoculation and if this finding can be applied to the generation of an efficacious vaccine for HIV-1

EXPERIMENTAL PROCEDURES

Mice and virus. Six- to eight-week-old female BALB/c and BALB/c nu/nu mice (Taconic Farms, Germantown, NY) were used for all experiments. RLV strain RVB3, derived from the original stock, was prepared by tail vein injection of 10^4 PFU of RLV into mice. Single cell suspensions of spleens obtained from animals sacrificed at 20 d were prepared in RPMI-1640 media (2 ml/g spleen) supplemented with 20% fetal calf serum and cell supernatants were prepared and stored in liquid N_2. The number of PFU in the stock was determined by XC plaque assay.

RLV inoculation and drug treatment. For the first sets of experiments, mice were inoculated with RLV and treated with AZT and IFN-α essentially as described (Ruprecht et al. 1990a, Hom et al. 1991). In brief, animals were iv injected with 10^4 PFU of RLV in a volume of 0.2 ml and, 3 h later, given 10^4 Units of recombinant human IFN-αA/D (Hoffman-LaRoche, Nutley NJ) (IFN-α), formulated in 0.1 mg/ml mouse albumin (Sigma Chemical, St Louis, MO) by i.p injection. Oral administration of AZT (0.1 mg/ml in drinking water) was also started at this time . Mice received single daily injections of IFN-α and given AZT for 21 d. For experiments without antiviral drug therapy, RLV inoculation doses were as shown.

Cyclosporin A (CsA) Treatment. The CsA oral stock solution (100 mg/ml in olive oil (Sandoz Pharmaceuticals, Hanover, NJ) was diluted further in light mineral oil (Sigma). A dose of 25 mg/kg/day in 50 μl was given i.p. for 21 d. Control mice received either 50 μl of a mixture of olive oil + mineral oil (1:10) or nothing.

Spleen cell populations. At the indicated time points after RLV inoculation, mice were sacrificed, the spleens were removed and then teased apart with forceps. Spleen

cells were isolated by centrifugation over Lympholyte-M, washed in RPMI-1640 with 10% FCS (assay medium), and either tested immediately for CTL assays or fractionated further.

For negative selection, spleen cells were incubated either with concentrated rat hybridoma supernatant GK1.5 (anti-CD4) or 53-6.72 (anti-CD8) (both from American Type Culture Collection) at 2 x 10^7 cells/ml in assay medium for 30 min on ice, washed twice and goat anti-rat Ig Immunomagnetic beads (Advanced Magnetics, Cambridge MA) were then added (0.6 ml original bead suspension per 2 x 10^7 spleen cells). After an additional 30 min on ice, the suspension was placed in a magnetic particle concentrator (Dynal, Great Neck, NY) and unbound cells were removed to a fresh tube and washed. For each depleted population, <3% of the cells stained positive for the respective CD4 or CD8 antigens as assessed by flow cytometry. For positive selection of either $CD4^+$ or $CD8^+$ spleen cells, Cellectplus immunocolumns (Biotex Laboratories, Edmonton, Canada) were used according to the manufacturers instructions. The purity of both the $CD4^+$ or $CD8^+$ T cells were >98%.

Cytotoxic T Lymphocyte Assays. RLV-infected target cells were prepared by incubating P815 cells (BALB/c mastocytoma cell line) with RLV (5 x 10^4 PFU/10^6 cells) in the presence of Polybrene (Sigma Chemical; 8 mg/ml) overnight at 37°C in culture media (Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 10 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin). Cells were expanded and stained for RLV Env and Gag antigen expression using anti-gp70 Env mAb 48, and anti-p30 Gag mAb R187 (both from ATCC) which were developed against Friend MuLV but cross react with RLV antigens (Chesebro et al. 1983). RLV-infected P815 cells were sorted by flow cytometry for Env expression, expanded and routinely checked for Env and Gag antigen expression which was maintained at >90%.

To measure specific cytotoxicity, RLV-infected or noninfected P815 cells were radiolabelled by incubation with $Na^{51}CrO_4$ (New England Nuclear; 100 μCi/10^6 cells) for 1 hr at 37°C then washed and incubated with the indicated effector cells in 96-well round bottom plates. After 5 h, supernatants were harvested onto SCS harvesting frames (Skatron Inc., Sterling, VA) and released ^{51}Cr measured in a g-counter. Assays were performed in triplicate and the percent specific cytotoxicity was determined according to the formula:

$$\frac{\text{(experimental release - spontaneous release)}}{\text{(total release - spontaneous release)}} \times 100$$

where spontaneous release is that from target cells cultured in medium alone and total release that from target cells cultured in 1% NP-40 detergent.

Primer Design and PCR. Isolation of mouse splens, DNA preparation and all PCR reactions were carried out wearing surgical gown, cap , and mask to avoid contamination. Each animal was dissected with a separate set of instruments. DAN from single-cell suspensions was isolated as published (Fazely et al., 1993). All PCR reactions with the exception of positive controls were assembled in a SterileGuard™ hood. Nested primer pairs were derived from the *env* region for the plus strand and from the U3 region of the 3'LTR for the minus strand. The first round primers were 5' CGTTGATATTCACCATCA3' (nt 1444 to nt 1461); 5'CGTTACTGCGGCTATCA3' (nt 1679 to nt 1663) and the second round primers were 5' GCATTCTTAATCAGGATCTCA3' (nt 1508 to nt 1528); 5' GCAACTTGGTGGGGTCGTTCA3' (nt 1656 to nt 1636). One to 4 μg genomic DNA were amplified according to the manufacturer's instructions using DNA amplification kits from Cetus (Perkin Elmer, Newton, MA) and an ErikompTwin Block™ thermocycler. To determine PCR sensitivity, different copy numbers of cloned Rauscher Spleen Focus-Forming Virus (R-SFFV) DNA (plasmid pBC10, Bestwick et al. 1984) were amplified

in the presence of 1 μg or 4 μg of genomic spleen DNA of naive mice. PCR reactions were covered using the Ampliwax™ system (PerkinElmer) in addition to hot start method to increase sensitivity. The temperature cycle program was as follows: 94°C for 1 min, 55°C for 90 sec, 72°C for 90 sec, all for a total of 35 cycles, followed by final extension for 10 min at 72°C. In comparison to light mineral oil which allowed detection of 1 copy per 10^4 cells using nested primers, the Ampliwax system with hot start resulted in a several-fold increase in sensitivity. During one round of PCR, 1-5 copies of pBC10 -plasmid DNA could be detecte din the presence of 4 μg of genomic DNA, corresponding to approximately 6 x 10^5 cells.

XC plaque assay. Live virus in sera from RLV-exposed animals were tested by XC plaque assay. In brief, on day -1, SC-1 cells (mouse fibroblast cell line)were cultured in 6 well plates (5 x 10^4 cell /well using cell culture media) and the following day, Polybrene (Sigma) was added to a final concentration of 8 μg/ml. One hr later, test sera or stock RLV was added. On day 1, wells were washed 2x and fresh media was added. On day 5, cultured SC-1 cells were killed by UV irradiation and XC-1 cells were added (5 x 10^5/well). Media was replaced on days 6 and 7 and the XC-1 cells were stained on day 8 with methyleneblue (0.33%)/carbol fuschin (0.17%) stain to visualize plaques. Sera were diluted with phosphate-buffered saline (PBS) 1:10 and filter sterilized before testing.

Immunoblot analysis for RLV. To obtain soluble samples, spleens were homogenized in RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, 1 mM phenylmethylsulfonylfluoride), and total protein of both spleen and serum samples was measured using Bio-Rad Protein Assay reagent (Bio-Rad, Hercules, CA). Twenty mg of total protein from serum or spleen samples were separated by 10% SDS-PAGE and

transblotted onto Immobilon-P membranes (Millipore, Bedford, MA). After blocking nonspecific binding sites with 0.1% Tween, membranes were incubated first with goat anti-RLV antiserum (NIH Repository Number 75S000294) and, after washing, with horseradish peroxidase-conjugated rabbit anti-goat IgG (Bio-Rad, Hercules, CA). Specific binding was demonstrated after the blot was developed with the substrate 4-chloro-1-naphhol (Life Technologies, Inc., Gaithersburg, MD). Individual lanes were scored positive for RLV by the presence of p30 gag, p15, and gp70 env bands.

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Table I

Vaccination with low-dose, live pathogenic RLV

	Group	Initial RLV Dose (AID_{50}) [a]	Fraction Virus Free [b]	RLV challenge (AID_{50}) [c]	Fraction Virus Free
Expt 1	1	2	3/10	200	2/3
	2	0.4	7/10	200	2/7
	3	0.2	10/10	200	0/10
	4	0.04	9/10	200	0/9
Expt 2	1	2	3/10	20	1/3
	2	0.4	7/10	20	1/7
	3	0.2	9/10	20	2/9
	4	0.04	10/10	20	1/10

a - Animals (10 mice per group) were inoculated iv with the indicated amounts of RLV on day 0 and serum was tested on day 21 for RLV antigens by immunoblot analysis.

b - The presence or absence of RLV in serum was confirmed by XC plaque assay.

c - Mice shown to be virus free following initial inoculations were challenged with acute doses of RLV and serum was reanalysed for RLV antigens 21 days afterwards.

Figure 1. *Ex vivo* RLV-specific CTL activity in AZT plus IFN-α treated, RLV-inoculated mice. Animals were given RLV inoculations (5000 AID_{50}) and started on combination anti-viral drug regimen as described in Materials and Methods. On the days indicated, mice were sacrificed and the CTL activity in purified spleen cells was measured immediately without stimulation or culture using RLV-infected target cells at an effector:target ratio of 200:1 The results show the CTL response from individual mice at each time point. Killing of control uninfected target cells was always <3% at the same effector to target ratio.

Figure 2. RLV-specific CTL activity following T cell subset depletion. Mice were inoculated with RLV and given AZT plus IFN-α as described in Figure 1. On day 21, spleen cells from individual mice were isolated and depleted of either $CD4^+$ cells or $CD8^+$ cells as described. Unfractionated (▲), $CD4^+$ depleted (+), and $CD8^+$ depleted (□) spleen cells from individual mice were then tested for RLV-specific CTL activity. Each graph shows the level of killing using spleen cells from an individual animal.

Figure 3. RLV-specific CTL activity of purified $CD4^+$ and $CD8^+$ T cells from AZT plus IFN-α treated, RLV-inoculated mice. Separate populations of $CD4^+$ and $CD8^+$ T cells were isolated by positive selection as described in Materials and Methods from spleens of mice 21 d after RLV inoculation and the start of combination drug therapy. CTL activity against RLV-infected target cells was then measured for each cell population at the indicated effector to target ratios.

Figure 4. Time course of RLV proviral sequence levels in splenic isolates following RLV inoculation and post-exposure anti-viral drug therapy. 4 mg of genomic DNA was amplified and 10 ml of PCR products were run on 2% agarose gels. Virus only: no drug therapy was given after RLV inoculation; Drug (+4 h) and (-3 d): combination therapy

(AZT + IFN-α) was given 4 h after and 3d before RLV inoculation, respectively. PBS only: negative controls given PBS but no virus or drugs. M: ϕX174 DNA digested with HaeIII as molecular marker.

Figure 5. CTL activity in spleen cells of immune protected mice after rechallenge with an acute dose of RLV. Animals which were shown to be free of virus following high-dose RLV inoculation and anti-viral drug therapy were rechallenged with 20 AID_{50} of RLV on day 28 after the initial RLV inoculation. CTL activity in spleen cells was then measured as described in Figure 1 on the days indicated after rechallenge at an effector to target ratio of 200:1. No CTL activity was detected after d5. Data shown are for individual mice at each time point.

Figure 6. Survival analysis of CsA-treated RLV infected mice. The probability of survival was analysed by Kaplan-Meier statistics and is given as a function of time (in days) post inoculation of mice (10 per group). Control groups received RLV (20 AID_{50}) only or RLV + oil.

Figure 7. Rauscher Murine Leukemia Virus (RLV) stock titration in normal and athymic nude mice. Either normal BALB/c or BALB/c nu/nu mice were inoculated i.v. with increasing concentrations of a stock of RLV and, 21 d post-infection, serum from individual animals was analysed for RLV antigens by immunoblotting. The values at each dosage point indicate the number of infected mice in each group over the number animals receiving the indicated dose.

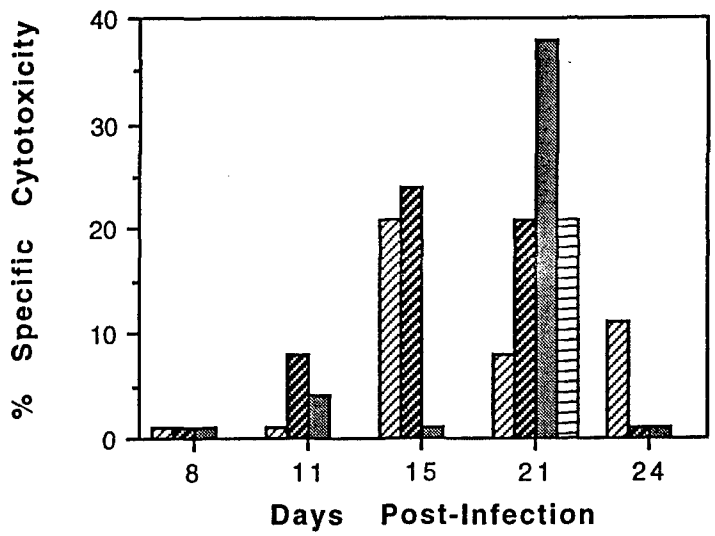
% Specific Cytotoxicity
40
30
20
10
0
8
11
15
21
24
Days Post-Infection

Figure 1

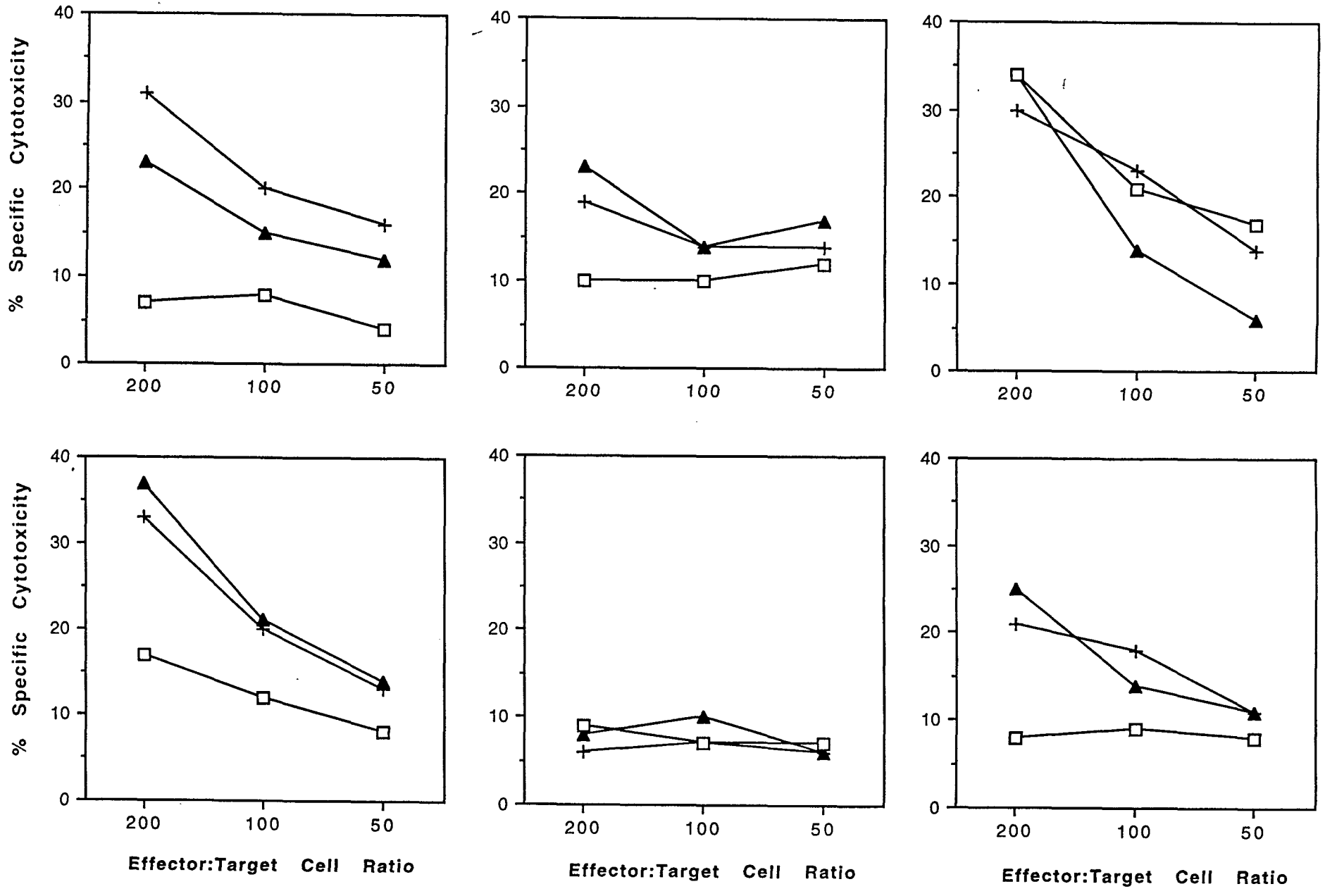
% Specific Cytotoxicity
40
30
20
10
0
200
100
50
Effector:Target Cell Ratio

Figure 2

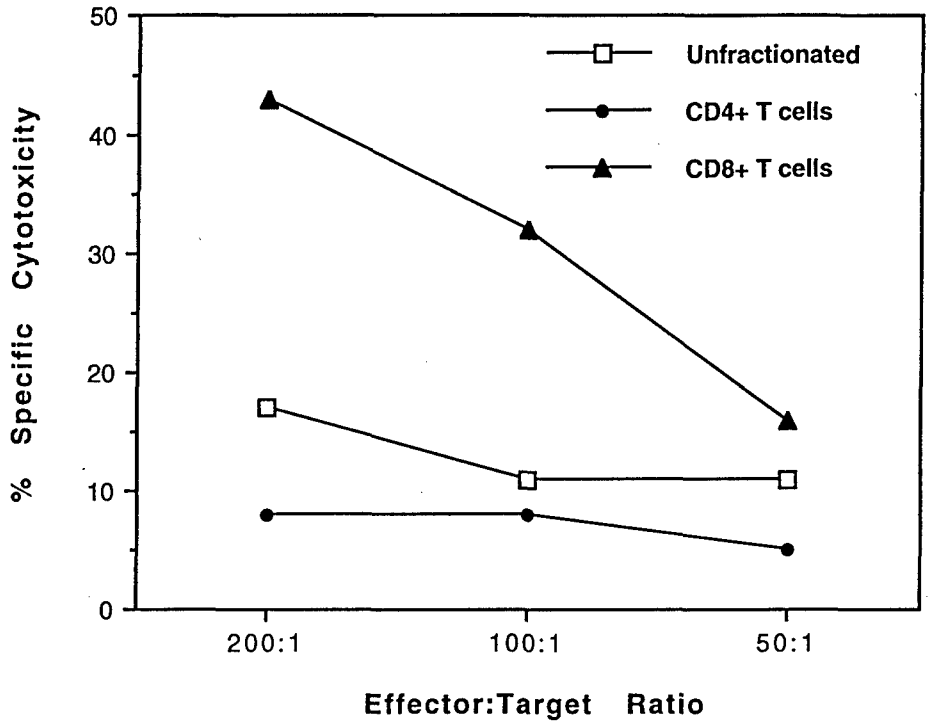
50
40
30
20
10
0
% Specific Cytotoxicity
Unfractionated
CD4+ T cells
CD8+ T cells
200:1
100:1
50:1
Effector:Target Ratio

Figure 3

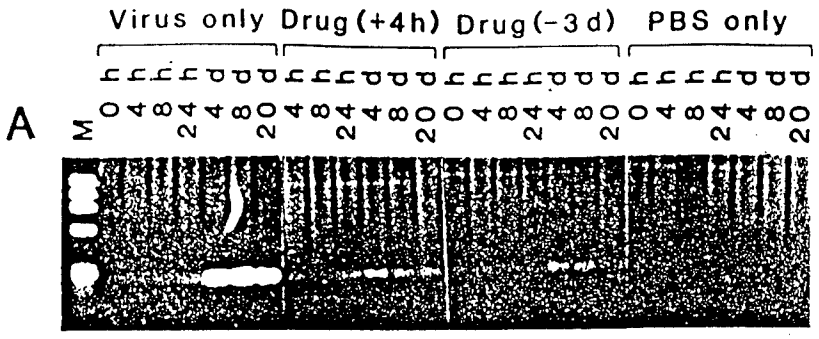
A
M
0 4 8 24 48 20
Virus only
0 4 8 24 48 20
Drug (+4h)
0 4 8 24 48 20
Drug (-3d)
0 4 8 24 48 20
PBS only

Figure 4

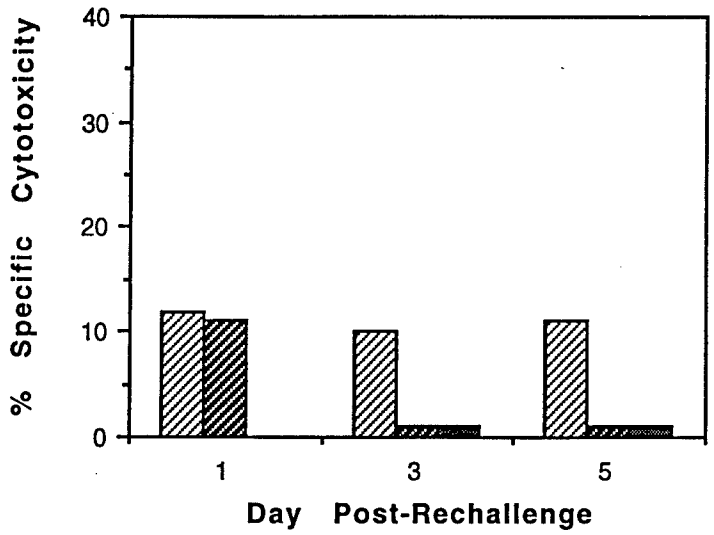
% Specific Cytotoxicity
40
30
20
10
0
1
3
5
Day Post-Rechallenge

Figure 5

rr00-0a1.n08 1/11/95:TA

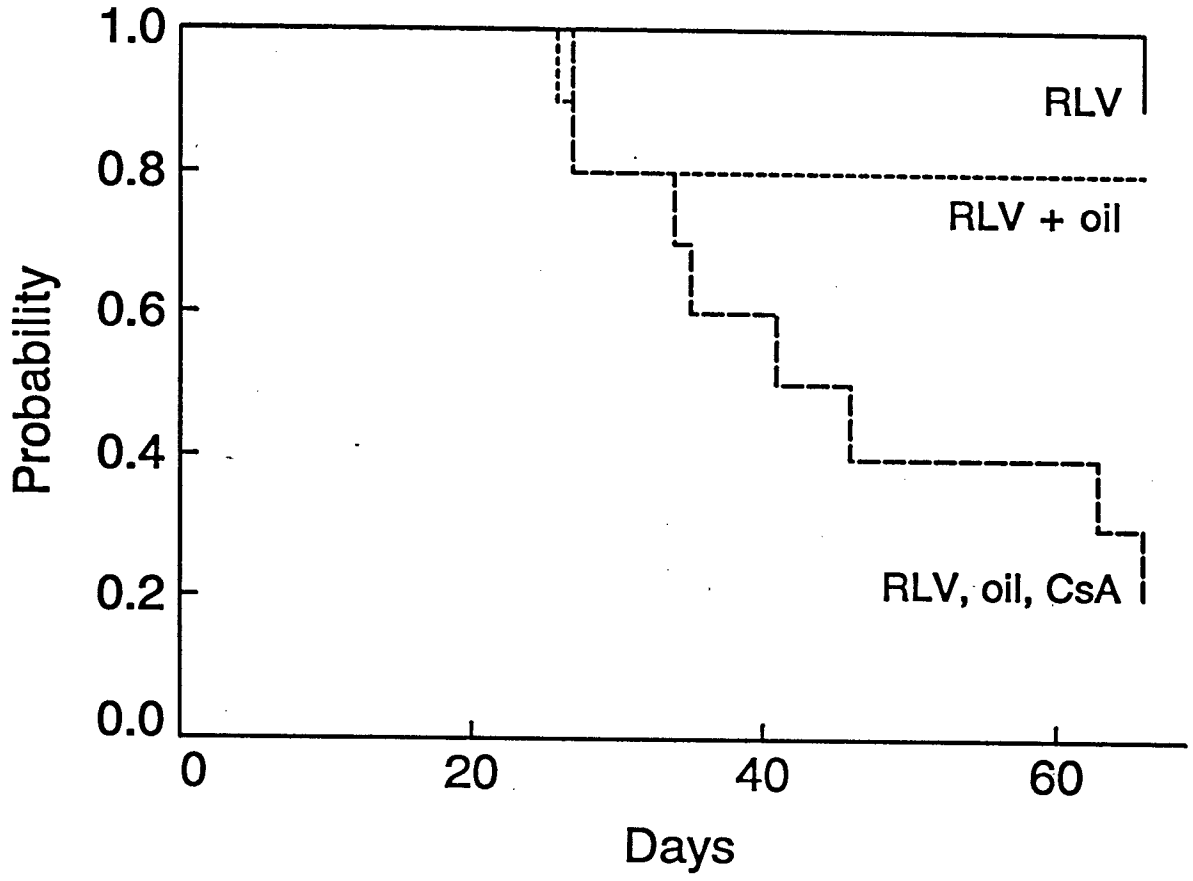

Figure 6

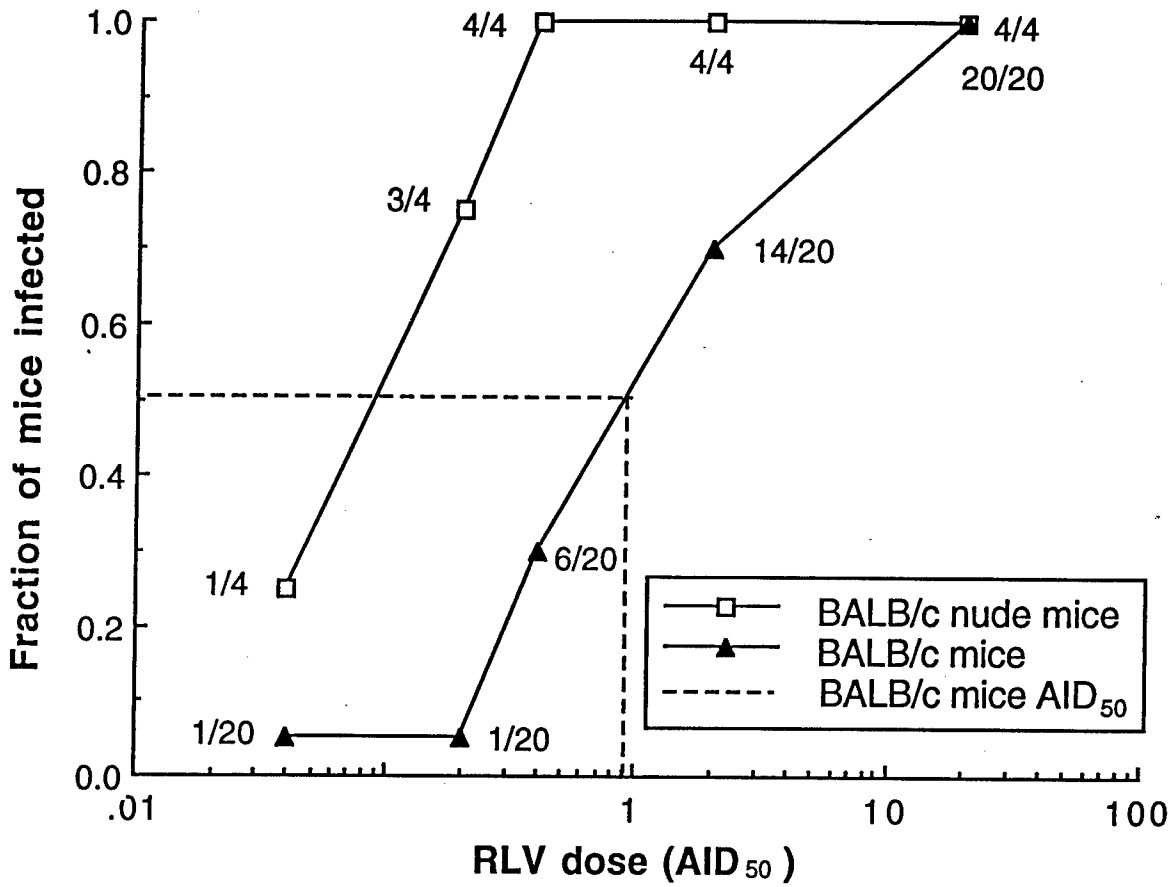
1.0
0.8
0.6
0.4
0.2
0.0
Fraction of mice infected
.01
1
10
100
RLV dose (AID50)
4/4
4/4
4/4
20/20
3/4
14/20
6/20
1/4
1/20
1/20
BALB/c nude mice
BALB/c mice
BALB/c mice AID50

Figure 7

Received 2/8/00

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